Inducible Nitric Oxide Synthase and Periodontal Inflammation: A Preclinical Canine Study.

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ABSTRACT

Harriet L. Arrington, DDS: Inducible Nitric Oxide Synthase and Periodontal Inflammation: A Preclinical Canine Study.
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Up-regulation of inducible nitric oxide synthase (iNOS) has been linked to periodontal disease and its progression. This preclinical study was therefore conducted to evaluate the effects of a selective iNOS inhibitor, INO-1001, on the initiation and progression of experimental gingivitis in the canine model. Following a safety and feasibility study, eighteen beagles were randomized to treatment with topically administered INO-1001 or placebo gels and experimental gingivitis was induced over an 8-week period. Clinical outcomes (i.e., plaque index, ginigival index, bleeding on probing and modified sulcular bleeding index) were measured at baseline, 4 and 8 weeks. No inter-group differences were noted at the baseline or 4-week examinations. At the 8-week exam, measures of gingival inflammation and sulcular ulceration were significantly increased in the INO-1001 group compared to placebo. These results suggest that iNOS may play a dual role in the pathogenesis of periodontal disease and may be critical for periodontal homeostasis.

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ADMA	- Asymmetric dimethylarginine
AG	- Aminoguanidine
AGE	- Advanced glycated end products
APC	- Antigen presenting cell
ASA	- Aspirin
ATP	- Adenosine triphosphate
BH4	- Tetrahydrobiopterin
BOP	- Bleeding on probing
b.i.d.	- Twice daily
CCD	- Charge-coupled device
cNOS	- Constitutive nitric oxide synthase
COX	- Cyclooxygenase
DNA	- Deoxyribonucleic acid
ECM	- Extracellular matrix
eNOS	- Endothelial nitric oxide synthase
FAD	- Flavin adenine dinucleotide
FDA	- Federal drug administration
FMN	- Flavin mononucleotide
GCF	- Gingival crevicular fluid
GED	- Guanidinoethyldisulfide
GI	- Gingival index
I-CAM	- Intercellular adhesion molecule

IFN-β	- Interferon beta
IFN-γ	- Interferon gamma
IL-1	- Interleukin -1
iNOS	- Inducible nitric oxide synthase
i.p.	- Intraperitoneal
i.v.	- Intravenous
LBP	- Lipopolysaccharide binding protein
L-NMMA	- N^{G}-monomethyl-$_{L}$-arginine
LO	- Lipooxygenase
LPS	- Lipopolysaccharide
LTB_4	- Leukotriene B_4
MCP	- Monocyte chemoattractant protein
MEG	- Mercaptoethylguanidine
MIP	- Monocyte inflammatory protein
MMP	- Matrix metalloproteinase
MSBI	- Modified sulcular bleeding index
NADPH	- Nicotinamide adenine dinucleotide phosphate
NFκB	- Nuclear factor kappa B
NLR	- Nod-like receptor
nNOS	- Neuronal nitric oxide synthase
NO	- Nitric oxide
NOS	- Nitric Oxide Synthase
NSAID	- Non-steroidal anti-inflammatory drug

PARP	- Poly-ADP ribose polymerase
PDL	- Periodontal ligament
PGE_2	- Prostaglandin E_2
PI	- Plaque index
PMN	- Polymorphonuclear leukocyte
p.o.	- Peroral
PRR	- Pathogen recognition receptor
RANK	- Receptor activator of nuclear factor kappa B
RANKL	- Receptor activator of nuclear factor kappa B ligand
RNA	- Ribonucleic acid
RNS	- Reactive nitrogen species
ROS	- Reactive oxygen species
s.c.	- Subcutaneous
SOD	- Superoxide dismutase
Tc	- T lymphocyte cytotoxic cells
Th	- T lymphocyte helper cells
TLR	- Toll-like receptor
Tm	- T lymphocyte memory cells
TNF-α	- Tumor necrosis factor alpha
Treg	- T lymphocyte regulatory cells
TXA_2	- Thromboxane A_2
VEGF	- Vascular endothelial growth factor

1. INTRODUCTION

Periodontal disease, encompassing both gingivitis and periodontitis, is a host-mediated inflammatory process initiated by oral bacterial insult which may result in significant alterations in the normal structure and/or function of the supporting tissues of the dentition. Although colonization of host tissues by pathogenic organisms is the initiating factor in this disease process, the associated rate of progression and degree of destruction are dependent upon both the virulence of the invading organisms and the magnitude/persistence of the host response to this infection.

The initial response of the human host to subgingival microbial infection is an innate immuno-inflammatory reaction primarily directed by gingival epithelial cells and polymorphonuclear leukocytes (PMNs). If this process is ineffective in the elimination of invading pathogens, then various immune and inflammatory cells are recruited to the area, a multitude of cytotoxic and inflammatory mediators are released, and multiple enzyme systems are activated, ultimately resulting in enhanced inflammation and tissue destruction. The amplitude of this inflammatory response is dependent upon the genetic profile of the microorganism and the host, as well as related environmental factors.[1] Depending upon the extent of inflammation and destruction, resultant changes in the periodontium may become irreversible. Moreover if left untreated, the disease process can lead to chronic inflammation, extensive compromise of periodontal support with resultant changes in the dentition, and repeated systemic exposure to periodontal pathogens.[2-4] This repeated systemic bacterial exposure and resultant inflammatory response may play a significant role, either directly or

indirectly, in the pathogenesis of many systemic diseases and their sequelae. To date, periodontal disease has been linked to a variety of systemic disease states and conditions including: cardiovascular disease[5,6], cerebrovascular disease[7,8], diabetes[9,10], the delivery of pre-term, low birth weight infants[11-13], and most recently, pancreatic cancer[14]. Due to the significant morbidity and mortality associated with these conditions, the importance of prevention, diagnosis, and effective treatment of periodontal disease may increasingly be appreciated from a systemic health perspective.

As can be recognized from the above introduction to periodontal disease, modification of host-pathogen interactions are of paramount importance in the prevention and treatment of this disease process as well as its subsequent sequelae. Historically the vast majority of treatments have focused on the subgingival microbiota and its elimination or alteration via mechanical or chemotherapeutic modalities. These interventions have been demonstrated to be quite effective in disease management; however, in a significant portion of the population they have proved inadequate in halting disease progression.[15] Thus, recent studies have primarily been directed at modification of the host's contribution to this disease process. The inhibition of a variety of key mediators have been investigated with several showing promise in preclinical and/or clinical trials. One such mediator, nitric oxide, is currently receiving much attention due to its association with multiple chronic inflammatory processes, including periodontal disease, and will be the principal focus of this thesis.

2. OBJECTIVES

The current study was designed with the objectives of: 1) evaluating the feasibility and safety of topical and systemic administration of three novel iNOS inhibitors in experimental gingivitis and periodontitis; 2) testing the hypothesis that over-expression of iNOS is detrimental to the periodontal tissues; and therefore, its inhibition should lessen the extent and severity of gingival inflammation in the canine model of gingivitis; and conversely, 3) testing the alternative hypothesis that iNOS is essential for periodontal homeostasis and serves a protective role during this disease process.

3. BACKGROUND AND SIGNIFICANCE

To understand the complexities of modifying host-pathogen interactions, a basic appreciation of the nature of the sub-gingival microbiota and host immune response is necessary. Therefore, a brief review of current knowledge regarding these topics, as well as a discussion of the collective and individual contributions of key cells and mediators to the pathogenesis of periodontal disease will be presented. A summary of key host modulatory therapies, pertinent findings and limitations will also be described with specific focus on inhibition of nitric oxide synthase, the enzyme responsible for nitric oxide production.

Although nitric oxide is essential for homeostasis and immunity, its production via the inducible isoform of nitric oxide synthase (iNOS) has repeatedly been implicated in periodontal disease and its progression. Furthermore, inhibition of nitric oxide synthesis in animal models of both experimental gingivitis and periodontitis has been shown to be beneficial.

3.1 PERIODONTAL MICROBIOTA

Although the relationship of plaque and calculus to periodontal disease has been appreciated since ancient times, the relationship of microbes to this picture was not recognized until the late 1600's. Anton van Leeuwenhoek in 1683 first described the microscopic presence of a vast array of "animalcules", or microorganisms, in dental plaque.[16]

Since this time, there has been significant interest in determining the nature of these microorganisms and their relationship with disease.

The contributions from multiple microbiologic studies in the 1960's and 1970's have greatly enhanced our understanding of the nature and composition of subgingival plaque. These studies described not only trends of compositional shifts in the microflora during the transition from periodontal health to disease, such as an increasingly Gram-negative, anaerobic, and motile profile, but they also noted that specific organisms appeared to frequently be associated with the presence of disease.[17-20] Furthermore during this time period, our knowledge of the structure of subgingival plaque was greatly enhanced by investigations of bacterial communities in other aqueous environments. Reports of the existence and early characterization of bacterial "biofilms" facilitated the recognition that sub-gingival plaque was not the chance accumulation of bacteria and debris, but instead, was the result of formation of highly structured bacterial communities.[21-23] It has subsequently been established that the sub-gingival bacterial biofilm forms via rather specific bacterial interactions and microbial successions.[24-26] This biofilm has been shown to exhibit nutritional co-operation, environmental modification (i.e., oxygen detoxification), small-molecule signal-mediated gene regulation, gene transfer, and enhanced resistance to host defenses and antimicrobials.[21-23]

Many studies have been conducted in the isolation and characterization of various subgingival microbes in an attempt to determine which bacteria are likely associated with the pathogenesis of periodontal disease. In 1998, Socransky categorized bacteria which had been isolated from subgingival plaque samples into groups, which not only loosely represented the sequence of colonization of these organisms in the periodontal pocket and their relationships

with one another, but also represented the relative association of the organisms with the pathogenesis of periodontal disease.[27] In Socransky's description of these subgingival microbial complexes, the "orange" and "red" complexes both consisted of organisms commonly associated with disease, with the "red" complex organisms being those most strongly related to clinical parameters of inflammation and periodontal destruction.[25] The organisms in the "orange complex" consist of *Fusobacterium nucleatum*, *F. periodonticum*, *Prevotella intermedia*, *P. nigrescens, Peptostreptococcus micros, Campylobacter rectus, C. showae, C. gracilis, Eubacterium nodatum, and Streptococcus contsellatus*, and these organisms predominantly constitute the loosely adherent plaque found between the tooth-associated and epithelial pocket-associated plaque in the periodontal pocket.[25,26] The "red complex" organisms are found to be spatially associated with the subgingival epithelium and comprise the majority of the pocket-associated plaque.[26] These organisms include *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia*.[25-28] Although not included in Socransky's model due to the population studied, *Actinobacillus actinomycetemcomitans* is also frequently associated with periodontal disease, especially localized aggressive forms, and has also been classified as a periodontal pathogen.[29]

The bacteria that are currently recognized as "periodontal pathogens" include *A. actinomycetemcomitans (Aa), P. gingivalis (Pg), and T. forsythia (Tf*).[29] These organisms have been consistently associated with periodontitis and its progression, and have been shown to possess a variety of factors which allow them to significantly modify the immuno-inflammatory response and promote tissue destruction.[29] These factors include components such as lipopolysaccharides, peptidoglycans, lipoteichoic acids, fimbriae, proteases, heat shock proteins, formyl-methionyl peptides, and cytotoxins.[30] These allow bacteria to adhere

to and invade host tissues, regulate the host immune response, and ultimately result in host defense evasion, bacterial persistence, and enhanced host tissue destruction.[30]

Although considerable progress has been made in characterization of the subgingival biofilm, much has yet to be understood. It has been estimated that over 400 different bacterial species may inhabit the subgingival periodontal pocket, and of these organisms, less than half have been cultured.[31] We, therefore, currently have a very limited picture of the true nature of the microbial composition, interactions, and virulence factors associated with the subgingival biofilm. Also, at this time, the mechanisms by which these bacteria interact with the host to elicit the immune response have not been fully elucidated. The recent discoveries of a variety of non-specific pathogen recognition receptors (PRRs) in mammalian cells has provided a greater understanding of the nature of host-microbial interactions.[32-34] These receptors have the capacity for non-specific recognition of potential pathogens based upon highly conserved molecular patterns present in a variety of microbial components. The PRRs in humans are currently known to include Toll-like receptors (TLRs), Nod-like receptors (NLRs), and helicase domain-containing antiviral proteins.[35] Although the roles of several of these receptors have yet to be fully defined, the types and quantities of mediators released during an infection will, in large part, depend upon the differential stimulation of these receptors. Due to expression of these PRRs by the majority of cell types, rather than only by those of myeloid or lymphoid origin, these findings have demonstrated a possibility for extensive interactions between the innate and adaptive immune response and have established a potential role for a variety of cells in the host immune response.

Although an appreciation of the subgingival bacterial biofilm is essential in understanding the pathogenesis of periodontal disease, it is insufficient to explain the

variability in disease expression and severity among patients. Host susceptibility, due to defects in one or more aspects of the host response, has been identified as an essential component for periodontal disease progression.[1] It has therefore been established that "the periodontopathic bacterial flora is necessary but not sufficient for disease."[1]

3.2 HOST RESPONSE IN PERIODONTAL DISEASE

Host response refers to the recognition, activation, and subsequent regulation of an individual's immune response to the presence of an antigenic or foreign substance. This response is generally an immuno-inflammatory process aimed at the eradication of the offending microorganism. However, the immune system of an individual also has the capability of suppressing this response which is important for preventing the recognition of "self" proteins as well as maintaining colonization by various beneficial, or commensal, organisms. Although currently not fully understood, this immunosuppressive mechanism, known as tolerance, is essential for the prevention of host cell recognition and inappropriate activation of the immune response. However, some pathogenic bacteria may take advantage of this mechanism to prevent host detection and permit invasion and replication within host tissues.

The first line of defense of the gingival sulcus, or crevice, against bacterial insult relies on both the structural integrity and physiologic nature of the crevice. The presence of an intact sulcular epithelium acts as a physical barrier in preventing the penetration of bacteria and bacterial products into the epithelium, underlying tissues, and systemic circulation. The gingival crevicular fluid (GCF), which is an exudate derived from the serum of adjacent blood vessels, aids in "flushing" bacteria and their products from the base of the

crevice outward into the oral environment. Also depending upon the level of disease present, the GCF may contain substances such as immunoglobulins, complement, cytokines, antimicrobial peptides, immune and bacterial cells, enzymes, and metabolic byproducts.[36] The turnover or shedding of the crevicular epithelium is also important in helping to prevent both bacterial colonization and invasion. Additionally in health, the termination of the junctional epithelium, which comprises the base of the sulcus, is at or above the cementoenamel junction. The tooth surface that opposes the sulcular epithelium is therefore composed entirely of enamel, which, in contrast to the cemental covering of the root surface, is considerably less plaque retentive and prone to bacterial contamination.[37, 38]

Assuming these primary defense mechanisms are adequate, no further response is required by the host; however, if the bacterial flora is sufficiently pathogenic, or the host is at an increased susceptibility to invasion due to various genetic or environmental factors, the help of phagocytic cells in the innate immune response is elicited.

In otherwise healthy individuals, the initial immune response to a pathogenic subgingival flora is one that is dominated by polymorphonuclear leukocytes (PMNs), or neutrophils. Even in health, PMNs are present in periodontal tissues and serve in a surveillance capacity, where they are found just beneath or migrating through the junctional epithelium into the sulcus. Although previously thought to only participate in the immune response via their barrier function, it has been demonstrated that the gingival epithelial cells are capable of recognizing pathogens via expression of PRRs (TLRs 2, 3, 4, 5, 6 and 9) and are able to produce and release β defensins, interleukin-8 (IL-8), IL-1β, tumor necrosis factor-α (TNF-α), IL-6, lipopolysaccharide binding protein (LBP), matrix metalloproteinases (MMPs) and nitric oxide based upon the microbial stimulus.[39-41] These mediators are able to

provide a strong chemotactic gradient to enhance the migration of PMNs into the gingival sulcus and activate other underlying cells such as mast cells, dendritic cells, macrophages, etc.[39-41] The infiltrating PMNs may become activated via direct interactions with bacteria and bacterial by-products or cytokines produced from resident cells. These activated PMNs are not only involved in the phagocytosis and killing of bacteria, but also release a variety of cytokines, chemokines, and inflammatory mediators, which are responsible for the further recruitment and priming of immune and inflammatory cells. In addition to PMNs, other local immune cells may also become activated via direct interactions with bacterial antigens or cytokines. According to the stimulus, these cells then release various pro-inflammatory and chemotactic molecules (i.e., TNF-α, interferon-γ, IL-1, IL-6, IL-8, IL-12, leukotriene B_4, NO, prostaglandin E_2, histamine, monocyte chemoattractant protein-1, monocyte chemoattractant protein-5, monocyte inflammatory protein-1a, and monocyte inflammatory protein-2) that, among other things, facilitate the infiltration of even larger numbers of immuno-inflammatory cells into the area. PGE_2, TNF-α, NO and histamine cause vasodilation and increase vascular permeability. The resultant compromise in vascular integrity aids in cell migration from the peripheral circulation as well as escape of intravascular fluid and proteins (i.e., immunoglobulins) into the surrounding tissues, resulting in edema and increased GCF production. In addition, the expression of E-selectin and P-selectin on the endothelium of the blood vessels and up-regulation of glycoproteins and intercellular adhesion molecule (I-CAM) on the PMNs facilitate diapedesis and migration of these cells out of the circulation and into the tissues. Furthermore, a family of enzymes known as the matrix metalloproteinases (MMPs) are produced and activated during this process. The MMPs are involved in the destruction of collagen and other extracellular matrix (ECM) components,

creating space for newly recruited immune cells. The recruited PMNs migrate through the junctional epithelium and densely aggregate along the surface of the sulcular epithelium. These PMNs primarily act to eliminate bacteria by means of phagocytosis. Additionally, the recruitment of monocytes and activation of macrophages play a significant role in bacterial phagocytosis and elimination in addition to the perpetuation of the inflammatory response. The production of complement and other acute phase reactants by hepatocytes, activation of the complement cascade and release of cytotoxic mediators also aids in bacterial clearance. Concomitant with this innate immune response, bacterial antigens recognized by TLRs on the dendritic cell may induce it to undergo a morphogenetic change, converting it from a stationary phagocytic cell to a mobile antigen-presenting cell (APC), which then can migrate to local and regional lymph nodes to activate the adaptive immune response. If bacterial clearance is achieved at this point, the immune response is quelled, and the local inflammation and tissue destruction are reversed. However, if this is not accomplished and the host response continues to escalate, the adaptive immune response with the associated activation of T and B lymphocytes begins to dominate. As this process continues, it becomes increasingly more complex with the involvement of a variety of cell types, the production and release of a multitude of mediators, and the contribution of various enzymes, mediators, and immune modulators by the pathogenic bacteria. Meanwhile, the zone and extent of tissue destruction increases.[1, 37-40, 42-44]

3.2.1 CELLS OF THE PERIODONTAL INFLAMMATORY RESPONSE

The cells active in periodontal disease pathogenesis are those that comprise the periodontium (epithelial cells, fibroblasts, osteoblasts, osteoclasts) and those classically

associated with the immune response (neutrophils, monocytes/macrophages, dendritic cells, lymphocytes). In an attempt to protect the host, these cells must maintain a delicate balance between pro- and anti-inflammatory actions, and any dysregulation in this balance may result in either an exuberant, host-destructive response or hypo-responsiveness that predisposes the host to infection.

Fibroblasts are the predominant cell type in the periodontium and are responsible for the normal synthesis and turnover of the extracellular matrix, within which the cells live and function.[37,38] This matrix is important for cellular migration, retention of pro-enzymes, growth factors, and other molecules involved in the response to tissue injury and wound healing.[37,38] One of the major components of the extracellular matrix is collagen, and the regulation of its synthesis and breakdown by fibroblasts is essential for periodontal health. Fibroblasts have been found to play an active role in host immunity and tissue destruction. They express TLRs 2 and 4 (PDL fibroblasts also express TLR-9) and respond to a variety of microbial factors in addition to multiple cytokines and mediators.[39, 40] Dependent upon the stimulus, these cells can produce chemokines (IL-8, MCP-1), cytokines (IL-1β, TNF-α), lipid mediators (PGE_2), nitric oxide, adhesion molecules (ICAM-1) and enzymes that degrade the extracellular matrix (MMPs).[39-41, 45, 46] In addition, gingival and PDL fibroblasts can express receptor activator of nuclear factor kappa B ligand (RANKL) following bacterial stimulation.[47-49] Due to the abundant nature of these cells and their ability to produce substances which have been highly linked to breakdown of the hard and soft tissues of the periodontium (IL-1β, TNF-α, PGE_2, MMPs, RANKL), fibroblasts likely play a critical role in the manifestations of periodontal disease as well as subsequent wound healing.[47, 50-54]

As briefly described above, PMNs, or neutrophils, are the first of the leukocytic immune cells to respond to infection and may kill bacteria or influence their growth via: 1) delivery of reactive oxygen and nitrogen species during "respiratory burst" 2) secretion of cytoplasmic granule contents, 3) phagocytosis by engulfment into phagosomes, or 4) death or apoptosis resulting in a release of intracellular contents.[37,55] The "respiratory burst" process has classically been viewed to involve the production of high levels of superoxide and other reactive oxygen species. However, it has lately been recognized that during this process NO is also produced by the iNOS enzyme and contributes to the ability of PMNs and macrophages to efficiently kill microorganisms.[41,56-58] In addition to their phagocytic function, these cells are also able to secrete cytoplasmic granule contents containing antimicrobial products and tissue destructive enzymes.[59] Neutrophils have been shown to possess all of the Toll-like receptors (with the exception of TLR-3), and accordingly are able to be stimulated by a wide variety of microbial products. The interaction of microbes, by-products, cytokines, and mediators with their corresponding cellular receptors determine the neutrophil's response (i.e., production of reactive oxygen species, NO, MMPs, IL-1, TNF-α, PGE_2, etc).[40] It has repeatedly been demonstrated that the proper functioning of these cells is essential for maintenance of periodontal health. Defects in neutrophil number, chemotaxis, adhesion, migration, bacterial killing efficiency, production of cytokines, NO, and enzymes have all been related to periodontal disease and tissue destruction.[60-65]

Macrophages constitute from 5 to 30% of the infiltrating cells in inflamed periodontal lesions.[42] Generally, low numbers of macrophages are recruited into the gingival sulcus; however, if neutrophils are inefficient in microbial clearance or bacteria/ bacterial products invade host tissues, the contribution of macrophages to the immune response increases.

Macrophages are phagocytic cells which have important functions in microbial clearance, antigen presentation, and enhancement of the inflammatory response. Monocytes/macrophages express TLRs 1, 2, 4, 5, and 6 and RANK on their surface and release an extensive array of chemokines and inflammatory mediators (IL-8, MIP-1, MCP-5, TNF-a, IL-1β, IL-12, IL-6, IFN-γ, PGE_2, NO).[39, 42, 66] Furthermore, these cells have been shown to have enhanced activity as APCs following exposure to RANKL, a mediator which is elevated in periodontitis lesions.[46, 67, 68] Similar to neutrophils, macrophages have also been implicated in host destruction. They have been shown to be the primary cells producing IL-1 in inflamed gingival tissues and a hyperinflammatory response by these cells has been noted in periodontitis patients.[69, 70] Due to the inflammatory nature of the macrophage response, these cells are thought to be important not only in periodontal disease, but also in the enhanced inflammatory response seen in obesity, diabetes, and atherosclerosis.[10, 71-73]

Dendritic cells are stationary phagocytic cells located beneath the epithelium which represent a major link between the innate and adaptive immune response. The expression of different TLRs upon dendritic cells is dependent upon their lineage (myeloid or plasmacytoid) and their location; however, upon stimulation, these cells may release various mediators, express co-stimulatory molecules on their surface, migrate to regional lymph nodes, present antigens to naïve T lymphocytes, and activate the adaptive immune response. Mature dendritic cells express a high level of RANK as well, which is thought to possibly enhance interactions between the dendritic cell and RANKL expressing T lymphocyte during antigen presentation.[40, 74]

The adaptive immune response is a lymphocytic response distinct from the earlier innate response by antigen specificity and memory. Adaptive immunity is subdivided into

cellular and humoral branches as follows. The cellular immune response is dominated by T lymphocytes while the humoral response is predominately a B lymphocytic response. Upon antigen presentation, the type of response elicited is dependent upon the nature of stimulus, co-stimulatory molecules present on the APC and cytokines/mediators released. The T lymphocytes are comprised by helper T cells (Th or CD4+), cytotoxic T cells (Tc or CD8+), and memory T cells (Tm). The helper T cells are further differentiated by the types of mediators that they release and type of adaptive response that they promote. The type 1 helper T cell (Th1) primarily releases IL-2 and IFN-γ and stimulates a Tc mediated response, the type 2 helper T cell (Th2) releases IL-4, IL-5, IL-6, IL-10, and IL-13 and stimulates the differentiation of B lymphocytes into plasma cells, whereas the type 3 regulatory T cells (Th3 or Treg) down-regulate the immune response. Cytotoxic T cells are involved with direct killing of infected host cells and thus, target intracellular pathogens, while B lymphocytes/plasma cells are of prime importance in the elimination of extracellular pathogens. Plasma cells produce antigen specific antibodies which neutralize bacterial products, activate the complement cascade, promote aggregation and clumping of bacteria, and promote opsonization of bacteria to enhance phagocytosis.[66]Although an effective adaptive immune response is critical for host health and survival, the presence of a chronic lymphocytic infiltrate is generally associated with periodontal tissue breakdown. It has classically been stated that the predominance of B cells/ plasma cells in the periodontium is associated with the conversion of gingivitis to periodontitis, and therefore, also to the initiation of alveolar bone loss.[75-77] This finding has recently been supported to some extent by the finding that over 90% of B lymphocytes in the inflammatory infiltrate of periodontitis lesions express RANKL, a potent stimulator of osteoclastogenesis. Although T lymphocytes

also express RANKL, a much lower percentage of cells have been shown to express this ligand.[46, 78] Another relatively recent finding that may further implicate lymphocytes in periodontal destruction is the presence of nictotine receptors on T and B lymphocytes.[79, 80]This finding may provide further understanding of the biologic mechanisms linking smoking and periodontal disease on both a local and systemic level. Despite multiple studies on the relationship between lymphocytes and periodontal disease, the roles of the different lymphocytes, their relative proportions, and the importance of their presence during different stages of periodontal disease are issues that remain quite controversial.

3.2.2 CYTOKINES AND OTHER INFLAMMATORY MEDIATORS

Cytokines are small protein mediators which constitute the principal mechanism of cellular communication. Cytokines released by cells are capable of acting in an autocrine and/or paracrine manner to influence cellular activity. Upon binding to their receptors, the actions of each of these signaling molecules will be dependent upon the cell and receptor which are stimulated, as well as the summation or cancellation of signals resulting from other mediators. The cytokines of greatest interest during the inflammatory response are IL-1, IL-6, and TNF-α.[42, 81]

The interleukins are a group of cytokines so named due to their production by, and involvement in, the cellular signaling of leukocytes. Although many interleukins play an important role in the immuno-inflammatory response via both pro- and anti-inflammatory mechanisms, the interleukins which have been most closely associated with an enhanced inflammatory response and subsequent tissue destruction are IL-1 and IL-6.

Interleukin-1 is a pro-inflammatory cytokine that is an important regulator of the host innate response and is recognized as one of the most significant mediators of tissue destruction in cases of periodontitis.[81] It was discovered in periodontally involved gingival tissues by Horton in 1972 and termed "osteoclast activating factor" (OAF) due to its ability to induce osteoclast activation and bone resorption.[50] OAF was later identified by Dewhirst to be interleukin-1β.[82] IL-1 is synthesized primarily by macrophages and monocytes; however, it is also produced by keratinocytes, dendritic cells, neutrophils and fibroblasts.[81] It is a potent stimulator of connective tissue catabolism and acts synergistically with TNF-α to stimulate bone resorption and inhibit bone formation.[54, 83] Additionally, IL-1 induces the release of large quantities of PGE_2 and MMPs by fibroblasts and monocytes.[81, 84]

Interleukin-6 is a cytokine which influences various aspects of the immune response and inflammatory reactions. It is primarily produced by activated monocytes, fibroblasts, and endothelial cells, and to a lesser extent by activated macrophages, T and B lymphocytes, osteoblasts and keratinocytes. Major functions of IL-6 include the induction of B cell maturation into plasma cells, stimulation of antibody secretion, and differentiation of cytotoxic T cells. IL-6 has also been suggested to play an important role in stimulation of osteoclastogenesis and bone resorption as well the induction of acute phase reactant production by hepatocytes (in conjunction with IL-1 and TNF-α).[42, 85, 86]

TNF-α is a pro-inflammatory cytokine that is primarily secreted by monocytes and macrophages. It induces the secretion of collagenase by fibroblasts, activates osteoclastic bone resorption, and induces the synthesis of IL-1 and PGE_2 in fibroblasts and macrophages.[42, 87-89] In addition to its previously mentioned synergistic actions with IL-1 and

IL-6 in the induction of bone resorption and production of acute phase reactants, TNF-α also acts synergistically with IL-1 and IFN-γ to potently induce nitric oxide production.[83, 90]

Other mediators of significance in the pathogenesis of periodontal disease include arachadonic acid metabolites, matrix metalloproteinases, and reactive oxygen and nitrogen species.

The arachadonic acid metabolites are lipid mediators derived from the cleavage of membrane phospholipids by phospholipase A_2 and subsequent enzymatic modification by lipooxygenase (LO) or cylcooxygenase (COX). These metabolites have been shown to play important roles in chemotaxis and mediation of the immune response. In the presence of lipooxygenase, arachadonic acid (AA) is converted to leukotrienes or hydroxyeicosatetraenoic acids (HETEs). However, in the presence of cyclooxygenase-1 or cyclooxygenase-2 (COX-1 or COX-2), AA undergoes enzymatic conversion to prostaglandin H_2 (PGH_2), which is then further metabolized to one of at least five prostanoids: PGE_2, prostaglandin I_2 (PGI_2 or prostacyclin), prostaglandin F_2 (PGF_2), prostaglandin D_2 (PGD_2), or thromboxane A_2 (TXA_2).[91] These prostanoids have a variety of biologic actions including effects on vascular tone, cardiovascular function, platelet aggregation, renal function, and bone turnover. Although cyclooxygenase-1 and cyclooxygenase-2 have similar biochemical activity in converting arachadonic acid to PGH_2 *in vitro*, the ultimate prostanoids they produce in vivo may be different due to differential regulation of COX-1 and COX-2, tissue distribution, and availability of the prostanoid synthases.[91] Prostaglandin E_2 and leukotriene B_4 (LTB_4) are two of the most highly implicated arachadonic acid metabolites in the progression of periodontal disease. LTB_4 is predominantly produced by activated neutrophils and is a potent chemotactic stimulus for the further recruitment of neutrophils and

monocytes. PGE_2 on the other hand is produced mainly by activated monocytes, fibroblasts, neutrophils and platelets and mediates vasodilation and vascular permeability, induces MMP secretion by monocytes and fibroblasts, and is a powerful inducer of osteoclastic bone resorption.[81, 84, 91-94]

MMPs are a family of zinc and calcium dependent enzymes that are responsible for the degradation of most extracellular matrix proteins during various physiologic processes such as fetal development, growth, tissue remodeling/repair and angiogenesis.[91] There are three major groups of MMPs: 1) the collagenases; 2) the gelatinases; and 3) the stromelysins.[91] These enzymes can be produced by a variety of cells (i.e., fibroblasts, neutrophils, keratinocytes, macrophages, endothelial cells, mast cells, etc.), and their activity can be regulated by natural inhibitors present in the tissues and serum (tissue inhibitors of metalloproteinases, α-macroglobulins). In general, MMPs are induced temporarily in response to exogenous signals such as various pathogenic substances, cytokines, growth factors, and cell matrix interactions. The expression and activity of matrix metalloproteinases in adult tissues is normally low, but is significantly up-regulated in various pathologic and inflammatory conditions.[91] As with other inflammatory processes, up-regulation of MMP expression has been demonstrated in patients with gingivitis and periodontitis.[95-97] TNF-α, IL-1, and PGE_2 appear to be the main signals for MMP synthesis by gingival fibroblasts, and subsequent host tissue destruction.[81, 91,98]

Reactive oxygen species (ROS), such as superoxide, hydroxyl radicals, hydrogen peroxide, and hypochlorous acid play an important role in the microbicidal activity of phagocytes and mediate a variety of signaling pathways.[55] Their endogenous production is primarily due to leakage from mitochondrial electron transport chain and NADPH-oxidase

activation during the respiratory burst process of phagocytic cells.[55, 99] The stimulation of superoxide production by PMNs and macrophages can be triggered by a variety of antigens, cytokines and mediators as well as the activation of Toll-like receptors by bacterial products.[99] Superoxide is a free radical which can rapidly cause cellular damage via oxidation of lipids and proteins.[99] Therefore, superoxide produced within the cell is quickly converted to hydrogen peroxide, a less toxic metabolite, by the enzyme superoxide dismutase (SOD). Superoxide dismutase has three principal forms, one found in the cytosol, one in the mitochondria, and one found extracellularly.[99] These enzymes have one of the highest reaction constants known in biologic systems which allows for the rapid dismutation of superoxide and prevention of cellular injury.[100] However, if the production of superoxide is excessive, SOD activity is decreased, or NO is produced in close proximity to superoxide, the reaction of these two molecules to form peroxynitrite, a molecule with even higher toxicity and potential for host tissue damage, may occur.[100]

Reactive nitrogen species (RNS) consist of the nitric oxide free radical and its products (nitric oxide, nitrite, peroxynitrite, nitroxyl and nitrosonium). NO is produced by a wide variety of cells and appears to be an important regulator of various physiologic processes in both animals and humans.[100] As with several other biologic mediators, this regulation appears to be affected not only by the presence of nitric oxide, but also by its relative concentrations and the presence of other mediators/cytokines. In addition, NO imbalances have been noted in a variety of chronic infectious and inflammatory conditions including periodontal disease.[65, 90, 101-103]

The above cytokines and mediators work in concert, and often synergistically, to up-regulate the local inflammatory response, induce the release of acute phase reactants from

hepatocytes, and enhance periodontal destruction. Elevated levels of these pro-inflammatory/ host destructive mediators (IL-1, IL-6, TNF-α, PGE_2, MMPs, ROS, RNS) have been found not only to be important in animal models, but have also been reported in the gingival crevicular fluid and gingival tissue samples of patients with periodontal disease.[53, 92, 97, 104-110] Greater elevations are generally noted in sites with active periodontal breakdown, while their concentrations following periodontal therapy have been shown to decline.[52, 111-121] The plausibility of periodontal destruction resulting from chronic high levels of these substances is further supported by the fact that bacteria of the subgingival biofilm have been shown to elicit their production in host cells. For example, lipopolysaccharide (LPS), a highly immunogenic component of Gram-negative bacteria, has been shown to be a potent inducer of IL-1, IL-6, TNF-α, PGE_2, MMP, ROS, and NO production via its interaction with TLR-4 which is expressed on the surface of cells comprising the periodontium as well as those involved in the immune response (epithelial cells, fibroblasts, osteoblasts/osteoclasts, endothelial cells, neutrophils, macrophages, dendritic cells, mast cells, etc.).[39, 40, 87]

3.3 NITRIC OXIDE: OVERVIEW

Nitric oxide (NO) is a gaseous, free radical, biologic signaling molecule which can readily diffuse through the cytoplasm and plasma membranes due to its solubility in both aqueous and lipid environments.[100] Its importance as a biologic mediator and the complex nature of its interactions have begun to be recognized in a multitude physiologic processes.[122-125] Most of the biologic actions attributable to NO are achieved by increasing intracellular cyclic guanosine monophosphate (cGMP) concentrations via binding and activation of soluble guanylyl cyclase; however, NO can also covalently bond with free thiol

groups of proteins and thiol containing free amino acids such as cysteine. Through the formation of nitrosothiols, NO may modify enzyme activity and possibly regulate various metabolic processes, including glycolysis and mitochondrial respiration.[100]

NO is generated within biologic tissues via the enzymatic conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). This enzyme exists in the body as three distinct isoforms: neuronal (nNOS or NOS-I), inducible (iNOS or NOS-II) and endothelial NOS (eNOS or NOS-III).[126] There is also some evidence that a fourth NOS isoform may be present in mitochondria (mtNOS); however, there is still considerable debate on its existence.[127-129] These isoforms have historically been categorized based upon differences in their primary locations in the body, inducibility, levels of nitric oxide production upon activation, and calcium dependence. Neuronal NOS and endothelial NOS are expressed constitutively, are calcium dependent and produce low levels of NO upon stimulation. Conversely, iNOS is regulated at the transcriptional level and is shown to be induced in multiple cell types upon bacterial or cytokine stimulation. Inducible NOS produces high levels of NO upon stimulation, reaches its maximal activity after approximately 24 hours, and is active at low calcium concentrations.[100] Despite these differences, the NOS isoforms are all quite similar in their structure, consisting of both oxygenase and reductase domains, and their requirement of L-arginine and key cofactors for activity (FAD, FMN, NADPH, calmodulin, tetrahydrobiopterin (BH4), and heme).[100] In response to insufficient availability of these factors (i.e., L-arginine or BH4 deficiency), an uncoupling of the oxygenase and reductase domains may occur and result in the production of reactive oxygen species such as superoxide and hydrogen peroxide.[100]

Nitric oxide has been found to have various roles in the normal physiology and pathophysiology of multiple biologic processes. It assumes a critical role in the regulation of vascular tone, smooth muscle proliferation, angiogenesis, coagulation, mitochondrial energy generation, neurotransmission, immunity, cell survival, and wound healing.[130-135] Conversely, nitric oxide as well as peroxynitrite have also been implicated in various inflammatory conditions such as arthritis, lupus, diabetes, cardiovascular disease, stroke, Alzheimer's disease, shock, multiple sclerosis and inflammatory bowel diseases.[136-138]

Most of the pathologies associated with NO are most often attributable to peroxynitrite, a powerful oxidant formed from the reaction of nitric oxide with superoxide. During infection and inflammation, significant quantities of both superoxide and nitric oxide can be produced by cells of the immune system.[100] When produced in relatively high quantities and in close approximation to one another, these molecules rapidly react to form peroxynitrite by a non-enzymatic reaction. Although superoxide dismutase (SOD), which is present in the cytoplasm, can rapidly breakdown superoxide, the rate of the reaction for the formation of peroxynitrite is approximately four times faster than the reaction of superoxide with superoxide dismutase.[100, 139] Formation of peroxynitrite can result in both oxidative and nitrosative stress since it consists of both a reactive oxygen and a reactive nitrogen species. Peroxynitrite has been shown to induce lipid peroxidation, damage proteins and nucleic acids, and result in DNA strand breaks.[140] Furthermore, it has been suggested that in an attempt to repair DNA damage, the enzyme poly-ADP ribose polymerase (PARP) is activated, resulting in depletion of nuclear adenosine triphosphate (ATP) stores and ultimately leading to cellular necrosis.[140] Since sufficient ATP stores are necessary for cells to undergo apoptosis, and thus limit the exposure of surrounding tissues to intracellular

enzymes, inflammatory mediators and toxins, the activation of PARP ultimately results in enhanced tissue destruction.[141-143]

Due to the ubiquitous nature of the biologic interactions involving NO and the apparent deleterious consequences of its dysregulation, the control of NO occurs upon many levels. Splice variants of all human NOSs have been found which decrease or obliterate NOS activity.[126, 144, 145] NO is regulated by feedback inhibition, the presence of endogenous NOS inhibitors such as asymmetric dimethylarginine (ADMA), and enzymes which compete for the substrate L-arginine (i.e., arginase).[129] Moreover, the availability of L-arginine and necessary NOS cofactors, the presence of compounds which readily bind NO (heme, glutathione), and the modulation of transcription and translation of NOS by deoxyribonucleic acid (DNA) methylation or by actions of various cytokines and mediators also are involved in the regulation of nitric oxide production.[100, 126, 146]

3.3.1 NITRIC OXIDE AND HOMEOSTASIS

Although the importance of nitric oxide has been demonstrated in a variety of biologic processes, those most pertinent to the discussion of periodontal disease include the effects of nitric oxide on the development, maintenance, and proper functioning of the bone, vasculature and immune response.

Evidence of the importance of NO in bone formation and remodeling has come from the observations of defective bone formation, volume, turnover, and osteoblast function in eNOS and iNOS deficient mice.[147-150] Interpretation of results from studies regarding the effects of nitric oxide on bone metabolism has been somewhat difficult in the past due to the presence and frequency of conflicting results. However, it is now understood that there is a

phasic response to NO in bone metabolism.[151] The presence of constitutive and inducible forms of NOS have been demonstrated in both osteoblasts and osteoclasts.[152] Osteoblasts have been shown to produce a low basal level of NO which is important for normal function. Significant increases or decreases in NO production via inflammatory stimuli or NOS inhibitors have been found to inhibit osteoblast proliferation and differentiation, and in some cases, result in apoptosis.[153-155] Low NO levels also appear to be important for the normal function of osteoclasts. If NO production is abolished or significantly increased, then bone resorption ceases under normal conditions. However, NO produced by iNOS also serves as an autocrine negative feedback signal that regulates osteoclastogenesis.[156-158] Furthermore, high NO levels appear to antagonize the effects of PGE_2 on bone resorption, whereas low levels appear to act in synergy in enhancing bone resorption.[159] Therefore, in the presence of inflammatory or pro-osteoclastic mediators, the inhibition of iNOS may result in significantly increased bone resorption.[151, 152]

The critical relationship of NO to vascular homeostasis was not recognized until the 1980's when Furchgott and coworkers discovered that the production of a chemical, which they termed "endothelium-derived relaxing factor" (ERDF), was the mechanism by which endothelial cells produced relaxation of vascular smooth muscle.[160] It was subsequently determined that ERDF was the gaseous free radical, nitric oxide.[123, 124] Since that discovery, research in the field of nitric oxide has increased exponentially. Both eNOS and iNOS have been demonstrated to be present in endothelial cells. Small amounts of eNOS have been found in platelets, and nNOS has been discovered in the nerve endings surrounding the vasculature as well as in the heart.[161-164] Studies have shown that NO inhibits vascular smooth muscle proliferation, platelet aggregation, down-regulates endothelial adhesion molecules,

mediates vasodilation, and enhances angiogenesis in conjunction with IL-8 and VEGF.[165-167] Furthermore, it has been demonstrated that the ability of VEGF to stimulate angiogenesis is significantly impaired in the absence of eNOS.[168]

Over almost two decades, the importance of nitric oxide in the immune response has been reported by numerous *in vitro* and *in vivo* studies. To date, nitric oxide's role in immunity has been most extensively studied in the murine model due to the ready availability, short gestational period, relative inexpense, and ease of handling of this small mammalian model. The effects of NO are also more easily elucidated in this model due to the ability to create NOS knockout models as well as the ability to rather quickly assess the effects of pharmacologic NOS inhibition on both the animals and their offspring. Although significant differences exist in human and murine physiology, studies have demonstrated similar roles for NO in the immune responses of both mice and humans. NO has been found to play an essential role in TLR signaling in mice and it has been demonstrated that iNOS knockout mice have an impaired response to bacterial infection in addition to a decreased susceptibility to endotoxic shock (a LPS/TLR-4-mediated process).[150, 169] Additionally, neutrophils and macrophages have been shown to possess both cNOS and iNOS, and the activity of these isoenzymes appears to be critical in the proper functioning of these cells (i.e., phagocytic activity, intracellular killing, and cell survival). Furthermore, a basal level of NO produced in neutrophils by cNOS has been suggested to be important in neutrophil chemotaxis via a cGMP dependent mechanism. The up- or down-regulation of this NO production has been shown to significantly impair neutrophil chemotaxis.[150, 170-172]

Researchers have also reported that nitric oxide plays an important role in the killing or containment of intracellular pathogens and parasites such as *Leishmania major*, *Giardia*

lamblis, and *Cryptococcus neoformans*; however, the addition of superoxide and subsequent peroxynitrite formation has been shown to reduce toxicity against these organisms likely due to decreased NO levels.[56-58, 173] In contrast, other microorganisms such as *Escherichia coli, Salmonella Typhimurium*, and *Candida albicans* appear to require the production of both oxygen and nitrogen free radicals by the host for maximal microbicidal activity.[173-176] Also, induction of iNOS by IFN-γ is believed to be essential for protection from viral infection.[177]

3.3.2 NITRIC OXIDE AND INFLAMMATION

Of the negative effects associated with nitric oxide, almost all are the result of an inflammatory process which is inappropriate in duration, severity or both. This inflammatory process leads to cellular destruction and ultimately results in tissue and organ dysfunction. One of the most frequently studied processes in which an acute up-regulation of iNOS is noted is that of septic shock. This is one of few acute disease states in which iNOS is implicated; however, this process gets much attention due to its high rate of mortality. During septic shock, the LPS of Gram-negative bacteria gains access to the systemic circulation and induces the production of massive quantities of NO, resulting in severe hypotension, poor tissue perfusion, organ failure, and ultimately death. There have been a number of studies which have implicated iNOS as the primary effector of this process, and still more have shown the benefits of NOS inhibition in animal models.[178-185] Multiple studies have also shown an increase in iNOS activity in chronic inflammatory diseases such as arthritis, inflammatory bowel disease, multiple sclerosis, and periodontal disease.[90,136-138,186] For example, serum and synovial fluid samples from patients with rheumatoid arthritis and osteoarthritis displayed enhanced formation of nitric oxide.[186] Furthermore, in

immunolocalization studies, iNOS was found to be strongly expressed in the synovial lining, fibroblasts, endothelial cells, and local inflammatory cells of inflamed joints while its expression was conspicuously absent in samples from healthy patients.[186-189]

Consistent with models of septic shock, NOS inhibition in animal models of arthritis, multiple slcerosis, and inflammatory bowel disease has also been found to diminish the associated inflammatory responses and suppress pathologic changes.[190-192] Additionally, in diabetic animals, NOS inhibition has been shown to prevent the formation of advanced glycation end products (AGEs), which through interactions with their respective receptors (RAGEs), perpetuate the inflammatory response.[193]

3.3.3 NITRIC OXIDE AND PERIODONTAL DISEASE

Substantial evidence also exists implicating NO in the pathogenesis of periodontal disease. LPS and other antigenic substances from putative periodontal pathogens such as *A. actinomycetemcomitans, P. gingivalis, P. intermedia, P. nigrescens*, and *F. nucleatum* have been shown to induce iNOS expression and NO production in murine macrophages.[194-202] It has been demonstrated by human *in vitro* studies that iNOS expression and activity is induced in gingival fibroblasts and neutrophils following stimulation by periodontal pathogens, cytokines, and other inflammatory mediators.[41, 203, 204] Also, neutrophils isolated from localized aggressive periodontitis patients were shown to display increased iNOS activity and subsequent chemotactic defects.[170] Using the rat model of experimental periodontitis, Lohinai and Di Paola have both demonstrated the presence a low baseline level of iNOS expression in gingival tissues, which increased significantly following ligature placement.[205, 206] These investigators also documented a parallel between the location and

degree of iNOS expression and the presence of 3-nitrotyrosine, a peroxynitrite reaction product, in gingivomucosal tissues of the study animals.[205, 207, 208] Additionally, Lohinai and colleagues demonstrated the absence of 3-nitrotyrosine and iNOS expression in the sterile gingival tissues of rat pups.[208] Similar studies examining the gingival tissues of human subjects with varying degrees of periodontal disease and periodontally healthy controls have also shown a strong relationship between iNOS expression, NO production, and the severity of inflammation and tissue destruction.[104-106, 209-211] Matejka and co-workers found that levels of L-arginine and L-citrulline, the precursors and by-products of NO synthesis respectively, were increased in the gingival tissues of patients with periodontitis as compared to healthy controls.[105] Others have demonstrated increased iNOS expression/ activity in diseased human gingival tissues and gingival crevicular fluid by measuring levels of nitrate/nitrite,[211] iNOS mRNA expression,[104] or iNOS protein expression via immuno-histochemistry.[106, 209-211] Utilizing this last method, Batista and co-workers quantitatively evaluated iNOS expression in gingival samples from patients with clinically healthy gingival tissues, plaque induced gingivitis, and localized chronic periodontitis.[210] Their findings demonstrated a significant increase in iNOS expression in gingivitis samples as compared to control. Expression was further increased in the samples from periodontitis patients.[210] The results of the above *in vitro* and *in vivo* studies cumulatively suggest that: 1) Host iNOS expression and activity in the periodontium is the result of persistent microbial insult, 2) NO and peroxynitrite are significantly linked to host inflammatory changes in the periodontium, and 3) NO may play an important role in both host defense and disease pathogenesis.

Following the presumption that iNOS up-regulation and subsequent peroxynitrite formation are detrimental to periodontal health via induction of DNA damage and PARP

activation, Lohinai and Di Paola have also investigated the involvement of PARP in periodontitis.[142, 205,212] In male rat models of experimental periodontitis, both investigators have found increased levels of PARP in diseased gingival tissues and have shown benefits of its inhibition in decreasing associated vascular permeability and bone loss using the PARP inhibitors PJ34 and 5-AIQ.[142, 205,212]

Equally important, several studies have demonstrated a protective function of NO, pointing to its role in bacterial killing and clearance of pathogenic organisms, as well as its possible anti-apoptic effects on PMNs.[150, 211] Gyurko and coworkers found that NO is an important component of the immune response against the periodontal pathogen *P. gingivalis*. In mice lacking iNOS, more severe infection developed and *P. gingivalis* clearance was decreased, despite enhanced production of superoxide by neutrophils. Additionally, they noted impaired PMN survival in the iNOS deficient mice.[150] This protective role was further emphasized in a study by Skaleric wherein increased GCF nitrate/nitrite levels were associated with reductions in *P. intermedia* colony-forming units (CFU) in diabetic patients with periodontal disease.[211] Carossa and colleagues in 2001 reported that oral NO production and salivary nitrite levels increased during *de novo* plaque deposition in periodontally healthy subjects. The authors proposed that this may be an early defense mechanism against bacterial proliferation in the dental plaque. They further demonstrated that this response was inhibited in smokers and was associated with an elevation in plaque bacterial counts as compared to non-smokers.[213]

4. HOST MODULATION IN PERIODONTAL DISEASE MANAGEMENT

Despite the many virulence factors and potentially host destructive compounds identified from periodontal pathogens, current evidence points to the host's immuno-inflammatory response as the primary cause of periodontal tissue destruction. More specifically, the dysregulation or over-expression of the earlier mentioned cytokines, enzymes, and inflammatory mediators have been repeatedly recognized in human and animal models of gingivitis and periodontitis. It is therefore reasonable that one of the areas of greatest research interest is in the modulation of inflammatory mediators for the treatment and prevention of periodontal disease. Primary avenues of host modulation which have been explored include inhibition of PGE_2 production, osteoclast inhibition by bisphosphonates, antagonism of IL-1 and TNF-α, inhibition of matrix metalloproteinases, the use of antioxidants/free radical scavengers, and inhibition of nitric oxide production.

4.1 INHIBITION OF HOST INFLAMMATORY MEDIATORS

Early studies on the modulation of periodontal disease by inhibition of specific pathology-associated host mediators (i.e., PGE_2) were conducted by Nyman and Williams in the 1970's and 1980's. Using canine models, due to similarities in periodontal disease presentation and progression in canines and humans, these investigators were the first to evaluate the effects of prostaglandin synthesis inhibition on experimental and naturally occurring periodontitis. They discovered that the systemic administration of non-steroidal

anti-inflammatory drugs (NSAIDs) was effective in slowing periodontal disease progression and inhibiting alveolar bone loss.[214-216] It has since been shown that a variety of NSAIDs (i.e., indomethacin, flurbiprofen, ibuprofen, ketoprofen, ketorolac, etc.) can lessen the extent of alveolar bone destruction and clinical attachment loss resulting from periodontitis.[217, 217-224] Furthermore, the mechanism of this protective action has been confirmed by GCF studies to be PGE_2 synthesis inhibition.[217, 225-228] It has long been recognized that aspirin and other NSAIDs exert the majority of their effects through the inhibition of the cyclooxygenase enzymes and thereby prevent the production of prostaglandins.[229] Due to the well established relationship between PGE_2 and periodontal disease, these drugs have received much attention. As a group, the NSAIDs have shown considerable promise and have been proven effective in topical and peroral formulations in both animals and humans.[217, 218, 230, 231] For example in the beagle dog, Paquette and co-workers demonstrated that topical administration of 0.03% and 0.3% S-ketoprofen gels significantly decreased the rate of alveolar bone loss and gingival index scores over a 60-day period. Similarly, in a 6-month study involving adjunctive NSAID treatment in chronic periodontitis patients, Jeffcoat and colleagues reported a reduction in alveolar bone loss and GCF PGE_2 levels in patients using a 0.1% ketorolac mouth rinse.[217]

The use of systemically administered NSAIDs in the treatment of periodontitis has been somewhat limited due to the inability to sustain long-term positive periodontal effects following NSAID withdrawal and concerns of tolerability and adverse systemic effects upon long-term systemic NSAID administration.[216, 232] Therefore, further studies of topical formulations and evaluation of emerging NO-NSAIDs, which are hoped to have a more favorable side effect profile, should prove beneficial.

Triclosan is an antimicrobial that also appears to have the ability to modulate the host response. In addition to its antibacterial effects, triclosan has also been shown to decrease PGE_2, IL-1β, and IFN-γ production in human fibroblast cell cultures.[233-236] It is currently believed that its effects on prostaglandin production are due to inhibition of prostaglandin E synthase production, rather than COX inhibition, the predominant mechanism of the NSAIDs.[234] Triclosan has been approved by the FDA for use in a dentifrice and has anti-plaque and anti-gingivitis indications.

Bisphosphonates are drugs commonly prescribed for the treatment of osteoporosis and other metabolic bone disorders. These medications inhibit osteoclast activity through the direct induction of osteoclast apoptosis as well as inhibition of farnesyl pyrophosphate, an essential enzyme for normal osteoclast function. Although bisphosphonates have shown some benefit in the prevention of bone loss associated with periodontitis, they have not been found to substantially improve clinical parameters of periodontal disease.[231] Furthermore, the chronic administration of these drugs for the treatment of periodontal disease raises concerns regarding therapeutic benefit versus risk of gastrointestinal irritation/ulceration and potentially significant systemic adverse events.

Due to the close association of IL-1 and TNF-α with inflammation and tissue destruction, modulation of these cytokines has been an area of interest for the treatment of various chronic inflammatory diseases, including periodontitis. In 1998, Assuma and colleagues evaluated the use of IL-1 and TNF-α antagonists (soluble receptors) in experimental periodontitis using *Macaca fasicularis* monkeys. They reported that these cytokine antagonists inhibited progression of the inflammatory front toward the alveolar crest and reduced the associated bone loss.[43] Further studies by this group have similarly shown

positive results and have demonstrated decreased osteoclast recruitment, connective tissue attachment loss, and alveolar bone loss following IL-1 and TNF-α inhibition (6μg/injection of each drug three times weekly for 6 weeks).[237-239] However, they have also demonstrated that these anti-cytokine therapies have negligible effects on gingival inflammation and result in delayed wound healing.[237-240] Additionally, in phase IV human clinical studies for arthritis and Crohn's indications, these drugs have been associated with a significantly increased risk for severe infection; and therefore should be used with caution.

Currently, of all the drug classes studied for the treatment of periodontitis via host modulatory effects, the anti-collagenolytics/MMP inhibitors have been the only class to have a drug approved for this indication. A sub-antimicrobial dose (20mg bid) of doxycycline, a member of the tetracycline antibiotics, received regulatory approval in the US in 1998 for the adjunctive treatment of chronic periodontitis. Tetracyclines, in addition to their utility in the treatment of bacterial infections, have also been shown to inhibit matrix metalloproteinases, and nitric oxide production independent of their antibiotic activity.[241-243] The adjunctive use of sub-antimicrobial dose doxycycline (SDD) has been demonstrated to significantly reduce loss of connective tissue attachment, improve probing pocket depth, decrease alveolar bone loss and decrease GCF collagenase levels in patients with chronic periodontitis, while not affecting subgingival microbial profiles or antibiotic resistance.[244-247] The inhibition of MMPs may also account for a portion of the actions of locally delivered tetracycline antibiotics as well. Among the currently approved tetracyclines, doxycycline appears to have the strongest anti-collagenase activity; however, multiple chemically modified tetracyclines are being developed which have even more pronounced effects on MMP inhibition.[248] Additionally, many of these drugs appear to inhibit osteoclast activity, prevent the release of

histamine and inflammatory mediators from mast cells, prevent the formation of advanced glycation end products (AGEs), and inhibit VEGF. Thus, this area holds much promise for the future treatment of periodontal disease.[249-251]

Another area that has received considerable interest lately in the treatment of acute and chronic inflammatory disorders is the modulation of nitric oxide production via nitric oxide synthase inhibitors and free radical scavengers. Agents that work only as antioxidants or free radical scavengers have proved to be of little benefit.[99] However, the inhibition of nitric oxide production has shown considerable promise in preliminary studies.

4.1.1 NITRIC OXIDE SYNTHASE INHIBITION AND PERIODONTAL DISEASE

Inhibition of nitric oxide synthesis has been proposed to be beneficial in the modulation of periodontal disease by preventing the participation of NO and other reactive nitrogen species in a variety of pathways which are thought to be deleterious to the host.[206] These pathways include modification of prostaglandin and cytokine production, induction of oxidative and peroxidative damage, activation of PARP, and ultimately depletion of cellular energy and cell necrosis (Figure 1).[206] Although non-selective inhibitors of nitric oxide synthase have primarily been studied in inflammatory conditions, inhibitors with iNOS specificity have recently been the main focus of investigation due to the documented up-regulation of iNOS in inflammatory conditions and importance of constitutive forms in homeostasis. In addition to inhibiting NO production, some NOS inhibitors, such as the alkyl guanidines and aminoguanidine, have also been shown to have alternate mechanisms of action.[252-254] The alkyl guanidines are a class of selective iNOS inhibitors, including mercaptoethylguanidine (MEG) and guanidinoethyldisulfide (GED), that have been

demonstrated to be potent inhibitors of iNOS while also simultaneously limiting peroxynitrite formation, scavenging available peroxynitrite and NO, and inhibiting prostaglandin production via inhibition of COX.[252-254] In diabetic models, aminoguanidine has been found to prevent the formation of advanced glycated end products (AGEs) *in vitro* and *in vivo*.[193] The formation of AGEs is of interest in periodontology since significant elevations in receptors for these products have been found in gingival tissues from diabetic patients with periodontitis in comparison to similar population groups who were periodontally healthy.[255-256] AGEs also elicit a hyperinflammatory response from macrophages and delay wound healing.[257-259] Therefore, inhibiting the formation of these end products should likely be of significant benefit in the prevention of systemic and periodontal pathology.

Although NOS inhibitors have yet to be studied in patients with periodontal disease, they have shown promise in animal models upon topical and systemic administration. In the modulation of experimental gingivitis in beagle dogs, Paquette and co-workers found that twice daily topical application of gels containing selective iNOS inhibitors, MEG (6mg) or GED (6mg), significantly reduced the clinical signs of gingival inflammation, including gingival index scores and bleeding responses at 8 weeks.[260] Other investigators have evaluated the effects of NOS inhibition in ligature-induced experimental periodontitis, with rather promising results. In 1998, Lohinai demonstrated in a rat model of experimental periodontitis that treatment with the iNOS inhibitor MEG, 30mg/kg intraperitoneally four times daily, reduced inflammatory extravasation and osteoclastic bone resorption at study termination on day 8.[206] Subsequently, Di Paola and colleagues reported that treatment with aminoguanidine, a semi-selective iNOS inhibitor, (100mg/kg i.p. daily for 8 days) reduced

neutrophil infiltration, lipid peroxidation, and presence of nitrotyrosine and poly(ADP-ribose) polymerase (PARP) in a rat periodontitis model. Additionally, they also found that aminoguanidine (AG) treatment resulted in decreased vascular permeability and alveolar bone destruction.[205] Most recently, Leitao and co-workers demonstrated that daily intraperitoneal administration of aminoguanidine and L-arginine methyl ester (L-NAME, a non-selective NOS inhibitor), using 5mg/kg and 20mg/kg respectively, significantly reduced the extent of the inflammatory cellular infiltrate and maxillary alveolar bone resorption in ligature-induced periodontitis in Wistar rats at 11 days. These investigators also evaluated several different dosages of the NOS inhibitors and noted that low doses of these NOS inhibitors (AG 2.5mg/kg and L-NAME 5 or 10mg/kg) did not significantly reduce bone loss; while high doses (AG 100mg/kg and L-NAME 40mg/kg) inhibited neither alveolar bone loss nor local inflammatory changes.[261]

As demonstrated by the previous studies, NO appears to play an important and rather complex role in the immuno-inflammatory process and in the remodeling and maintenance of osseous structures. It is therefore logical that modulation of this mediator has potential in the treatment of a number of inflammatory conditions including periodontal disease. Conversely, with the biphasic pharmacodynamics of NO, insufficient or excessive inhibition of iNOS may exacerbate the inflammatory process.

5. ANIMAL MODELS OF PERIODONTAL DISEASE

In experimental medical studies, the choice of animal model is an important decision and is based on a variety of factors such as cost, accessibility, ease of housing and care, animal docility, and similarity to humans. The canine has been recognized as a standard higher animal model for the study of gingivitis and periodontitis since the 1970s. Furthermore, this model has been extensively used in the testing of new interventions and chemotherapeutic modalities for the treatment of periodontal disease.[218, 262-267]

The similarity of animal models with humans is essential for the determination of relevance of findings and extrapolation of results to human subjects. The most commonly used animal models for the study of periodontal disease have been the rodent, canine, and non-human primate.

As would be expected, the rodent model, most frequently the rat, is the least expensive, and most easily obtained; however, there are significant differences in the anatomy and physiology of the dentition and periodontal tissues between rats and humans. Naturally occurring periodontal disease in this model is rare, and there are a number of differences in the oral flora and elicited immune response.[267]

Conversely, primates are the animals which have the greatest degree of genetic and phenotypic homology with humans. Their teeth and periodontal structures are very similar to those of humans and they have been shown to exhibit naturally occurring periodontitis both in captivity and in the wild.[267] However, these animals are very difficult to handle, are costly to obtain and house, and are rarely necessary for early trials.[267]

In contrast, the canine has proved to be a very useful model in the study of periodontal disease. This is due in part to similarities in size and structure of the dentition and supporting periodontium to that of humans.[267] Furthermore, this model (specifically that of the beagle dog) has been shown to have naturally occurring gingivitis and periodontitis in the presence of supra and subgingival plaque and calculus accumulations.[267] Additionally, their immune response has been shown to be similar to that of humans. A study by Lindhe and Rylander in 1975 reported clinical and histologic gingival alterations in beagle dogs following the cessation of oral hygiene measures.[263] The authors found that the initial plaque formation and accumulation was accompanied by progressive increases in gingival crevicular fluid flow and clinical signs of inflammation which was in accord with the studies of experimental gingivitis in humans.[263,268] In histologic assessment, they also demonstrated an absence of a connective tissue inflammatory cell infiltrate at baseline which gradually increased with time and shifted from a PMN dominated lesion to one composed of increasing levels of plasma cells and other mononuclear leukocytes.[263] Support for the canine as an appropriate model for the study of periodontal disease also comes from a recent study by Radice and co-workers which provides further evidence of the similarity of the subgingival flora between canines and humans. They found that in naturally occurring periodontitis in the canine, the most frequently isolated anaerobes included *Bacteroides fragilis, P. gingivalis, and P. intermedia*.[269] Other studies have also shown a strong relationship between the presence of *T. forsythia* and *P. gingivalis* with attachment loss in ligature-induced periodontitis in this animal model.[270] Support for the canine model for the study of host modulatory therapies comes not only from the histologic and clinical similarities between humans and canines in periodontal disease presentation and progression, but also from the

noted involvement of key inflammatory mediators in both groups (i.e., PGE_2).[226] Furthermore, the periodontal benefit exhibited with of inhibition of these mediators has similarly been documented in humans and canines.[218, 219, 226]

6. STUDY DESCRIPTION

Due to the documented involvement of inducible nitric oxide synthase in the immuno-inflammatory process, its up-regulation in chronic inflammatory conditions, including periodontal disease, and the purported benefits of NOS inhibition in animal models of periodontal disease, the current study was designed with the objectives of: 1) evaluating the feasibility and safety of topical and systemic administration of three novel iNOS inhibitors in experimental gingivitis and periodontitis; 2) testing the hypothesis that over-expression of iNOS is detrimental to the periodontal tissues; and therefore, its inhibition should lessen the extent and severity of gingival inflammation in the canine model of gingivitis; and conversely 3) testing the alternative hypothesis that iNOS is essential for periodontal homeostasis and serves a protective role during this disease process.

6.1 SPECIFIC AIMS:

Phase I: Safety/ Feasibility

1) To assess the safety and feasibility of topical and systemic (peroral and subcutaneous) administration of three novel iNOS inhibitors, designated WW-85, IMS, and INO-1001, in canine models of experimental gingivitis and periodontitis.

2) To collect preliminary data on the effects of these drugs on clinical signs of inflammation and radiographic progression of alveolar bone loss in experimental gingivitis and periodontitis.

Phase II:

To specifically evaluate the effects of the selective iNOS inhibitor, INO-1001, on the modulation of experimental gingivitis in the canine model.

6.2 METHODS:

After obtaining protocol approval by the University of North Carolina Institutional Animal Care and Use Committee, the following randomized, placebo controlled study was initiated. The investigation was blinded and was conducted in two distinct phases. A total of 30 female beagle dogs, having received no antimicrobial or anti-inflammatory agents within previous 6 months, were included in the study. All animals were approximately 1 year of age with permanent dentitions. Upon arrival, animals were inspected by a veterinarian, housed in cages, and fed Sensible Choice canned dog food (Royal Canin, Inc., St. Charles, MO, USA) and water ad libitum. During pre-treatment scaling/polishing and all subsequent clinical examinations, the animals were anesthetized using 0.2 mg/kg acetyl promazine maleate, 15-17 mg/kg thiopental sodium, and 0.11 mg/kg atropine. Following sedation, all animals were monitored for post-operative recovery by the research team. Daily intraoral inspections were performed for evidence of oral mucosal reactions during the treatment period. In addition, animals were weighed bi-weekly to monitor weight gain/loss and to insure adequate feeding. Any animal that experienced signs of weight loss or adverse outcome due to study procedures was to be examined by the veterinary staff and, if appropriate, withdrawn from the study.

Phase I : Safety/Feasibility

Phase I of the study (safety and feasibility of topical and systemic iNOS inhibitors) involved 12 dogs (3 per treatment arm), and consisted of four segments: 1) pretreatment, 2) experimental gingivitis, 3) recovery, and 4) ligature-induced periodontitis.

Pretreatment:

During the pretreatment phase all dogs were brought to optimal health with thorough mechanical scaling and tooth polishing, followed by daily rigorous tooth brushing. Tooth brushing continued over a 14 day period until all beagles exhibited low inflammation scores (i.e., mean gingival index < 0.5).[271]

Experimental Gingivitis:

At the conclusion of the pretreatment period, beagles were randomized via random number table to one of four treatment arms: 1) INO-1001 0.1mg/ml gel, 2) IMS 120mg/ml gel, 3) WW-85 0.1mg/ml gel, or 4) placebo gel. All gels were formulated by Inotek Corporation (Beverly, MA) and were packaged into masked color-coded syringes.

During the 8-week period of experimental gingivitis, all oral hygiene measures were discontinued and the animals were maintained on a plaque-promoting diet consisting of canned dog food and water. Two milliliters (ml) of the randomized study formulations were applied to the buccal gingiva twice daily. Upon dosing, each beagle was held and its lips reflected while the gel formulation was expressed around the buccal surfaces of all premolar teeth. Care was taken not to disrupt any developing plaque deposits during these procedures.

Clinical plaque formation and gingival inflammation were assessed at baseline, and at weeks 2, 4, 6 and 8 during gel treatments. Clinical measures were recorded at distobuccal,

buccal, and mesiobuccal sites for premolar teeth only and included gingival and plaque indices.[271, 272] Also, the presence (percent) and extent of bleeding (modified sulcular bleeding index) to gentle periodontal probing was determined using a manual UNC-15 periodontal probe.[273, 274] A single masked examiner was pre-calibrated to assure greater than 90% absolute intra-examiner reliability, and performed all clinical index evaluations.

Animals were weighed bi-weekly throughout the treatment period. At pretreatment and week 8, 5.0 ml of blood was collected from each animal and analyzed to ascertain electrolyte status, renal and hepatic function, acute myocardial infarction enzymes and metabolic bone disease indicators.

Recovery/ washout:

The beagles were allowed to recover from investigational procedures for a minimum of 6 weeks prior to initiation of experimental periodontitis. During this time, all dogs were brought back to gingival health by mechanical scaling and daily tooth brushing.

Ligature-induced periodontitis:

To induce experimental periodontitis, 4.0 silk ligatures were placed around mandibular premolar teeth and oral hygiene measures were ceased.[214, 264, 265, 275] Upon initiation of the ligature-induced periodontitis phase, beagles were again randomized to one of four treatment arms: 1) INO-1001 30 mg/kg peroral (p.o.) once daily 2) IMS 30 mg/kg p.o. once daily 3) WW-85 3 mg/kg subcutaneous once daily, and 4) placebo p.o. once daily.

Radiographic examinations were conducted at baseline, week 4 and week 8. At the 4-week exam, ligature retention was checked and any loose or missing ligatures were replaced. Using customized occlusal stents, standardized, intraoral, periapical films were obtained at each time point. Stents were fabricated with the use of Regisil vinyl polysiloxane impression

material (Dentsply Caulk, Milford, DE) and Rinn XCP bite blocks (Dentsply International, Elgin, IL).[276] The source to object distance was fixed such that effects of magnification could be easily calculated. Care was also taken to minimize angulation errors by aligning the area of interest/ film perpendicular to the source beam and paralleling the XCP arm with a fixed vertical reference. Direct digital radiographs were obtained using a CCD (charge-coupled device) and a personal computer. All radiographs were exposed at 60 Kvp and 4 mAmps for 1 second and analyzed using appropriate software. Following acquisition, digital images were processed for enhancement of geometric standardization and contrast correction via Ruttimann algorithm: Subtraction radiography was then utilized to assess areas of alveolar bone density loss.[277, 278] Vertical linear measurements were made adjacent to the tooth roots at each interproximal site and mid-furcally. Again, individual body weights were recorded biweekly throughout the treatment period, and 5.0 ml of blood was collected from each animal for laboratory assessment at pretreatment and at week 8.

Phase II: Evaluation of INO-1001 in Experimental Gingivitis

For the second phase of the study, the protocol was amended to concentrate on the evaluation of topically administered INO-1001 versus placebo in an appropriately powered cohort with experimentally induced gingivitis. Using the gingival index (GI) as the primary outcome, the sample size was calculated *a priori* assuming normally distributed means. It was determined that a sample size of 9 beagles per treatment group would allow for detection of significant differences coinciding with a 0.18 stardard deviation and a 0.3 difference, at an α level of 0.05 and a β level of 0.10. (The standard deviation used was a previously reported value for placebo treated canines during an experimental gingivitis study.)[279] Since two groups were planned for the study, eighteen female beagles (9 per treatment arm) were

included. The pre-treatment and experimental gingivitis protocol used in Phase I was also followed in Phase II, with the exception of the use of a new, more bio-adherent formulation of topical INO-1001 gel. Clinical indices of plaque formation and gingival inflammation were assessed at baseline, week 4, and week 8 during experimental gingivitis. Clinical measures were recorded at distobuccal, buccal, and mesiobuccal sites for premolar teeth only and included gingival and plaque indices.[271, 272]

Again, animals were weighed bi-weekly throughout the treatment period. At baseline and week 8, 5.0ml of blood was collected from each animal and analyzed to ascertain electrolyte status, renal and hepatic function, acute myocardial infarction enzymes and metabolic bone disease indicators.

6.3 STATISTICAL ANALYSIS

For all numeric analyses in this study, the dog was considered the primary unit of analysis and outcomes were averaged across the animal and time point. Outcome scores for each group were represented as a mean ± the standard deviation (sd), and in all instances where appropriate, the data were further analyzed for determination of statistical significance using SAS/STAT statistical software (SAS Corporation, Cary, NC).

Due to the preliminary nature of phase I of the study, and consequent small sample size per group, the analysis was limited to descriptive statistics (i.e., means and standard deviations). For phase II results, all differences in mean clinical indices between groups were analyzed for significance at the 0.05 level using general linear models (GLM). Additionally, GI and MSBI scores were dichotomized to yield severity scores (i.e.,

percentage of sites scoring a 2 or 3) and were further analyzed for significant intergroup differences. [280]

7. RESULTS

Phase I:

During the study, adverse events were monitored and no clinically significant abnormalities were noted individually or among groups in regard to blood chemistries, hematologic profiles, body weights, or oral mucosal ulceration/ pathology (mean weights are presented in Figure 2). However, early during phase I experimental periodontitis, all animals in the WW-85 treatment group were withdrawn from the study following local abscess formation at the subcutaneous injection site (dorsal cervico-thorasic region). Upon examination of the WW-85 injectable formulation, no bacterial growth was observed; however, contamination with an unidentified solid particulate was noted. Also during this phase of treatment, one dog from the placebo group expired while under general anesthesia at the week 4 examination. An autopsy was subsequently performed; however, with the exception of a possible untoward effect of the general anesthetic, no causative factor could be determined and no overt pathology was observed.

Phase I mean gingivitis and plaque scores (PI, GI, BOP, MSBI) are summarized in Table 1. All groups displayed an increase in mean plaque and gingival indices between the baseline and week 4 examinations; these scores were further increased by the week 8 exam, confirming the effectiveness of the established methods for induction of experimental gingivitis. An unexpected tendency for increased gingival inflammation and sulcular ulceration, as evidenced by bleeding scores, was observed in the iNOS inhibitor treated groups, and most notably the WW-85 group.

Regarding phase I experimental periodontitis, the mean radiographic bone loss measurements (displayed in Figure 3) demonstrated increasing bone loss between baseline and week 8 for all groups secondary to ligature placement. Similar to our observations in the experimental gingivitis phase, animals dosed with iNOS inhibitors appeared to show greater periodontal disease progression as compared to placebo-dosed animals. However, these results should be interpreted with caution due to loss of one placebo animal prior to study completion and decreased ligature retention in the remaining placebo animals. The percentages of ligatures retained for IMS, INO-1001, and placebo treated groups respectively were 100%, 66%, and 50% at week 4 and 100%, 100%, and 50% at week 8. It therefore can be reasoned that any comparisons with this control group would have the potential to be considerably biased.

Phase II:

In concordance with phase I, no adverse events or clinically significant abnormalities were noted individually or among groups in regard to blood chemistries, hematologic profiles, body weights, or oral mucosal ulceration/ pathology.

The mean outcomes of phase II experimental gingivitis are presented in Figures 4-7. Once again, all groups displayed increases in mean plaque and gingival index scores from baseline to week 4; these scores were further increased by week 8. No significant differences were noted between groups for any of the clinical outcome measures at the baseline or 4-week exams. However by week 8, treatment with the iNOS inhibitor, INO-1001, resulted in more pronounced gingival inflammation as evidenced by significantly higher mean scores for GI, BOP, and MSBI in comparison to placebo ($p<0.0001$). This enhanced inflammation was noted in the absence of any significant differences in mean plaque index scores between

groups at week 8. The INO-1001 treated group displayed a mean BOP difference of 34.9% when compared to control (49.7% vs. 14.8%); this represented an approximate 236% elevation over placebo. Similarly, in regard to the modified sulcular bleeding index, placebo treated animals had a 366% lower mean score than those treated with INO-1001 (0.24 vs. 0.88). The mean GI scores for the INO-1001 and placebo groups were 1.39 and 0.98, respectively. Although not as marked as the differences noted in BOP and MSBI, the difference in the mean gingival index between groups was found to be significant (0.41, a 42% increase over placebo). Furthermore, when GI and MSBI scores were dichotomized to yield severity scores, significant differences among the groups were once again noted. The severity scores for GI, 39.6 vs. 7.7, and MSBI, 38.8 vs. 9.5, in the INO-1001 versus placebo groups respectively were found to be over 4-fold higher in the INO-1001 treated group. All above mentioned findings at the 8-week time point were found to be significant at the $p<0.0001$ level.

8. DISCUSSION

Over the past decade, many studies have been conducted to elucidate the role of nitric oxide in the immuno-inflammatory response and characterize its potential involvement in the pathogenesis of periodontal disease. Numerous reports have established the presence of inducible nitric oxide synthase in gingival tissues and have demonstrated its subsequent up-regulation in humans and animals with periodontal disease.[104-106, 205, 206, 209, 210] Furthermore, the benefits of nitric oxide synthase inhibition in the modulation of the gingival inflammatory response and inhibition of alveolar bone loss in animal models have been repeatedly documented.[205, 206, 260, 261] These findings, however, are in marked contrast to those of the present study which demonstrated an enhanced inflammatory response upon inhibition of inducible nitric oxide synthase with the agents tested. This observed progression of periodontal disease with the intended inhibition of iNOS activity was consistently noted in all phases of the investigation (i.e., phase II results were concordant with phase I findings of higher gingival inflammation scores and alveolar bone loss means). The results of the current study therefore support a possible dual role for iNOS in the periodontal disease process and suggest that iNOS may be essential for periodontal homeostasis.

In attempting to understand the inconsistencies between the results of the present study and those that have previously been published, an appreciation of the presence and locations of the nitric oxide synthase isoenzymes within the periodontal tissues is necessary. In 1998, Lohinai and co-workers described the localization and potential function of the three

NOS enzymes (nNOS, iNOS, eNOS) in the periodontium based upon animal studies.[207] This classic report provides the framework for the following discussion of NOS enzyme localization. It has been demonstrated by several groups that the gingival epithelium expresses iNOS with most activity being localized to the basal keratinocytes and intraepithelial macrophages.[41, 209] Low levels of enzyme expression have even been observed in the tissues of periodontally healthy patients, leading to the presumption that iNOS may be constitutively expressed or continually induced in the gingival epithelium. This type of expression is seen in the epithelium of the nasal mucosa and the paranasal sinuses and is thought to be a protective mechanism against persistent microbial challenge.[90] Just beneath the epithelium exists an extensive vascular plexus in which the endothelial cells display both eNOS and iNOS.[207] Surrounding this plexus is an intricate network of nNOS containing nerve fibers and eNOS and nNOS positive mast cells.[281, 282] It has been demonstrated that endogenous nitric oxide production and treatment with nitric oxide donors act to stabilize mast cells, preventing their degranulation and release of inflammatory mediators.[282, 283] It is therefore believed that the constitutive NO produced by the adjacent nerve fibers and endothelium may act to stabilize the mast cells of the periodontium. Endothelial NOS and iNOS are expressed by the gingival and PDL fibroblasts and local phagocytic cells within the connective tissue (macrophages and neutrophils express all three of the NOS isoenzymes).[41,152,170] In the underlying alveolar bone, osteoblasts and osteoclasts possess both iNOS and eNOS, while osteocytes express both eNOS and nNOS.[284-286] As has been demonstrated, there is extensive overlap in the expression of the three NOS isozymes within the cells and tissues of the periodontium; therefore, it is possible that alterations in one

isozyme (i.e., iNOS) may result in untoward effects due to compensation or dysregulation of the others (i.e., eNOS and nNOS).

Lohinai and Di Paola have both reported the substantial presence of constitutive NOS (cNOS: eNOS and nNOS) expression in the gingival tissues of rats, which was down-regulated following the up-regulation of iNOS.[205, 206] In streptococcal cell wall-induced arthritis in rats, McCartney-Francis demonstrated the up-regulation of iNOS, eNOS and nNOS protein expression in diseased versus healthy joints.[287] The administration of L-NIL (3 mg/kg i.p. daily), a selective iNOS inhibitor, resulted in decreased mRNA and protein expression for iNOS and a slight up-regulation of eNOS mRNA expression. The up-regulation of cNOS activity in the absence of iNOS was found to result in an increase in the inflammatory infiltrate and "extreme tissue destruction including extensive loss of bone and cartilage" by day 24.[287] This response was markedly worse when compared to diseased joints in which iNOS had not been inhibited. Conversely, administration of the non-selective NOS inhibitor, L-NMMA (30 mg/kg i.p. daily), significantly decreased inflammatory joint pathology.[287] Furthermore, a study of orthodontic tooth movement in a rodent model demonstrated that non-selective NOS inhibitors significantly delayed tooth movement; whereas an iNOS specific inhibitor had no effect, implicating eNOS and nNOS in osseous destruction.[288] Neutrophils and macrophages with altered expression of iNOS or cNOS have been noted to cause both chemotactic defects and overproduction of superoxide which may result in increased susceptibility to infection or increased tissue damage.[150,170-172,287,299] Additionally, while abolishing or significantly increasing NO production has been shown to inhibit osteoclastic bone resorption, low levels of NO production (which could potentially be produced by increased nNOS or eNOS expression in inflammatory lesions) have been shown

to act together with PGE_2 to enhance bone resorption.[156-159] From these reports it can be inferred that cNOS expression may be more highly associated with tissue destruction than iNOS; and iNOS may actually serve in a protective role.

NO acts as a feedback inhibitory signal for its own production.[159] Since iNOS induction results in greatly elevated NO production for an extended duration, it would be reasonable to postulate that its induction could result in deactivation and subsequent down-regulation of eNOS and nNOS.[205] This may actually serve to limit angiogenesis and vascular permeability in chronic inflammatory conditions since eNOS has been determined to be the isozyme predominantly responsible for the NO-mediated actions of vascular endothelial growth factor (VEGF), a potent factor for the induction of angiogenesis and vascular permeability.[168, 289] VEGF production has been shown to be induced in gingival fibroblasts by the periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans*, and has been reported to be increased in gingival tissues of periodontal patients and diabetics.[290-294] It has also been demonstrated that non-selective NOS inhibitors inhibit both VEGF-induced vascular permeability and vascular proliferation while the lack of iNOS activity alone does not.[295-298]

Inducible nitric oxide synthase has also been demonstrated to be important in TLR signaling, and iNOS deficiency has been shown to impair clearance of *P. gingivalis*, decrease PMN survival and enhance tissue damage.[150, 169, 300]

Therefore, potent selective iNOS inhibition as potentially observed in the present experiment may result in enhanced cNOS expression/activity, increased host mediated periodontal destruction, impaired host immune response and reduced bacterial clearance. This could subsequently enhance the vascularity, vascular permeability and edema in

gingival tissues, while also allowing for increased direct and indirect pathogen-mediated tissue destruction.

Although the proposed mechanisms outlined above may aid in understanding the results of the current study, they are unable to adequately resolve the inconsistencies between the findings of the current study and those previously reported. These inconsistencies may reflect publication bias toward studies demonstrating positive results; however, they may also be explained by the use of non-selective or semi-selective NOS inhibitors in previous investigations. [205, 206, 261, 301] Thus, inhibition of not only iNOS, but also eNOS and nNOS may have been responsible for the observed beneficial effects.[205, 206, 261, 301] Additionally, studies that used selective iNOS inhibitors may have shown positive effects in the modulation of periodontal disease as a result of COX inhibition, peroxynitrite scavenging, or incomplete iNOS inhibition.[206,260]

Before drawing conclusions from the presented results, the potential limitations of the current study should first be assessed. One aspect of the study design that may be viewed as a potential limitation is its relatively short duration of 8 weeks. Other experimental gingivitis studies in this model, however, have shown markedly increased measures of gingival inflammation by the 8-week time point with only relatively minor changes being noted in gingival index measures thereafter.[219,266,279] Studies by Howell and Paquette have also demonstrated a diminution of benefits derived from host modulatory therapy beyond the 8-week time point, likely due to the presence of an overwhelming bacterial burden.[219, 266] Therefore, although seemingly short, this study duration was selected due to the pronounced gingival inflammatory and host modulatory responses generally noted in this model by week 8.

Another potential limitation of the present study is that it was designed to evaluate clinical rather than biochemical effects of INO-1001 in experimental gingivitis. Because histologic or biochemical evaluations of the gingival tissues/GCF were not conducted, the mechanism by which INO-1001 exerted its effects in this study cannot definitively be proven.

Also, in assessing the validity of the study results, an evaluation of the outcomes for the placebo group in comparison to other studies is warranted. Although the mean score for bleeding on probing in the placebo group at week 8 is lower than that previously reported by Paquette and colleagues in 2006 (i.e., BOP~ 15% vs. 32%), the current study was initiated with a lower baseline level of gingival inflammation (i.e., GI~ 0.19 vs. 0.3 and BOP~ 3% vs. 15%); and therefore, this variation is reasonable.[260] Also, the mean GI, BOP, and MSBI scores reported here for placebo-treated animals at baseline and week 8 are in agreement with those of similarly designed studies published by Howell and colleagues.[219, 279]

9. CONCLUSIONS

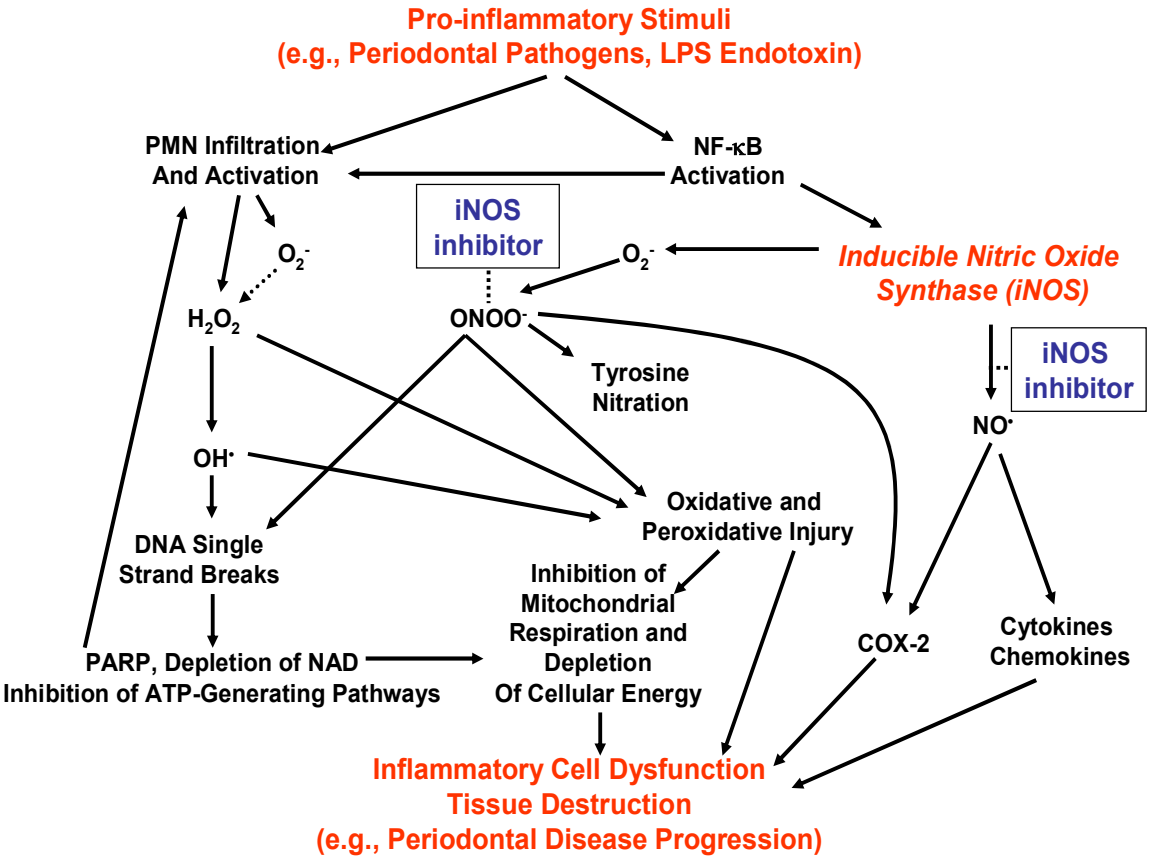
In an assessment of safety and toxicity, the results indicate that the selective iNOS inhibitors IMS, WW-85, and INO-1001 in topical and peroral forms appear safe and produce no mucosal reactions or systemic events in the canine model.

Regarding the effects of iNOS inhibitors in the modulation of experimental periodontitis, the selected inhibitors tested in this preclinical investigation, IMS 30mg/kg and INO-1001 30mg/kg, appeared to increase periodontal disease progression in the beagle dog. However, caution should be used in the interpretation of these results as comparisons are made to a biased placebo group, secondary to reduced ligature retention and attrition of one animal in this group.

In contrast to the previously reported benefits of NOS inhibitors in decreasing gingival inflammation, the present investigation found that selective iNOS inhibition with the agent INO-1001 appeared to exacerbate the extent and severity of experimentally induced gingivitis in the canine model. These results suggest that iNOS may be essential for periodontal homeostasis and may serve a protective role during this disease process.

Figure 1

Proposed mechanism by which iNOS inhibitors prevent cellular injury and tissue destruction



Adapted from Lohinai et al 1998 &
Rosenberg 2002

Table 1

Phase I - Experimental Gingivitis:

Mean Outcomes by Treatment Group

(Mean +/- Standard Deviation)

Treatment Groups (n= #animals/ group)		Plaque Index	Gingival Index	% Bleeding on Probing	Modified Sulcular Bleeding Index
Placebo	*Baseline*	0.04 +/- .13	0.13 +/- .23	4.1% +/- 1.1	0.05 +/- .16
n=3	*Week 4*	1.81 +/- .35	0.88 +/- .32	27.6% +/- 4.9	0.54 +/- .56
	Week 8	2.25 +/- .43	1.06 +/- .24	17.9% +/- 7.9	0.36 +/- .46
INO-1001	*Baseline*	0.17 +/- .29	0.14 +/- .25	4.8% +/- 1.9	0.07 +/- .24
n=3	*Week 4*	2.17 +/- .29	0.98 +/- .21	11.1% +/- 5.6	0.22 +/- .38
	Week 8	2.53 +/- .44	1.17 +/- .26	27.0% +/- 4.5	0.54 +/- .57
IMS	*Baseline*	0.21 +/- .32	0.18 +/- .29	11.1% +/- 4.1	0.17 +/- .30
n=3	*Week 4*	2.25 +/- .29	0.99 +/- .25	16.7% +/- 7.8	0.35 +/- .54
	Week 8	2.60 +/- .33	1.03 +/- .24	24.6% +/- 13.8	0.51 +/- .50
WW-85	*Baseline*	0.10 +/- .24	0.13 +/- .22	11.1%+/- 2.3	0.12 +/- .19
n=3	*Week 4*	2.17 +/- .33	1.09 +/- .32	27.8% +/- 5.9	0.61 +/- .50
	Week 8	2.58 +/- .44	1.26 +/- .29	38.9% +/- 2.3	0.79 +/- .66

Figures 2a & 2b

Figure 2a Mean Pre & Post Treatment Weights
Stratified by Treatment Group
Phase I: Experimental Gingivitis

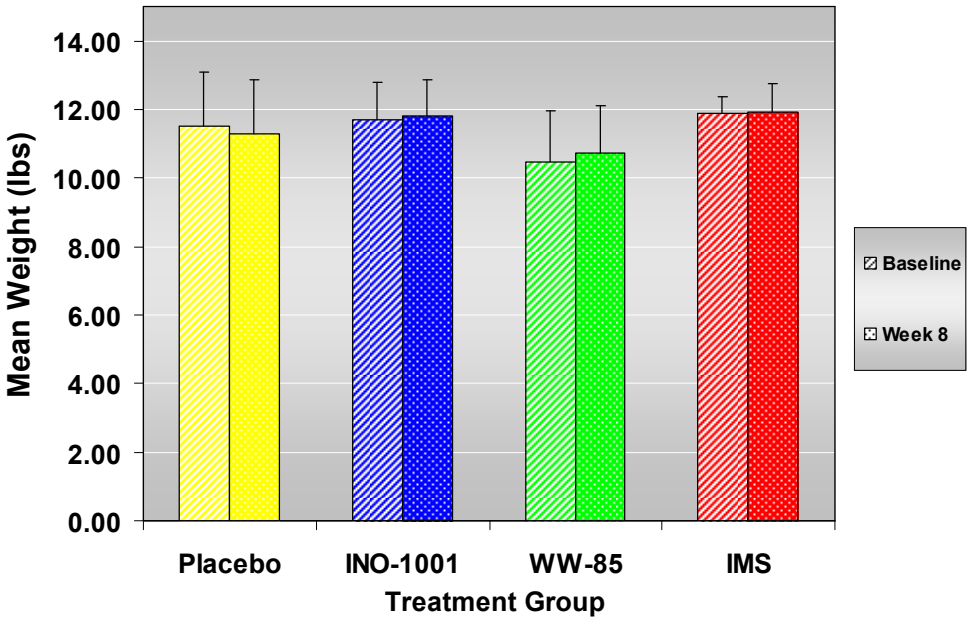


Figure 2b Mean Pre & Post Treatment Weights
Stratified by Treatment Group
Phase II: Experimental Gingivitis

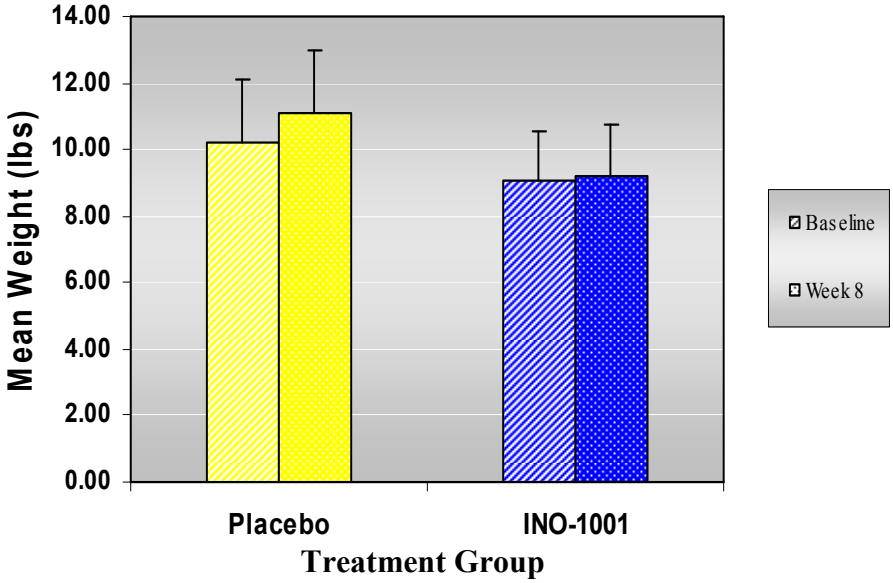


Figure 3

Phase I - Experimental Periodontitis Outcomes:
Mean Radiographic Bone Loss (in millimeters) Stratified by Treatment Group and Time Point

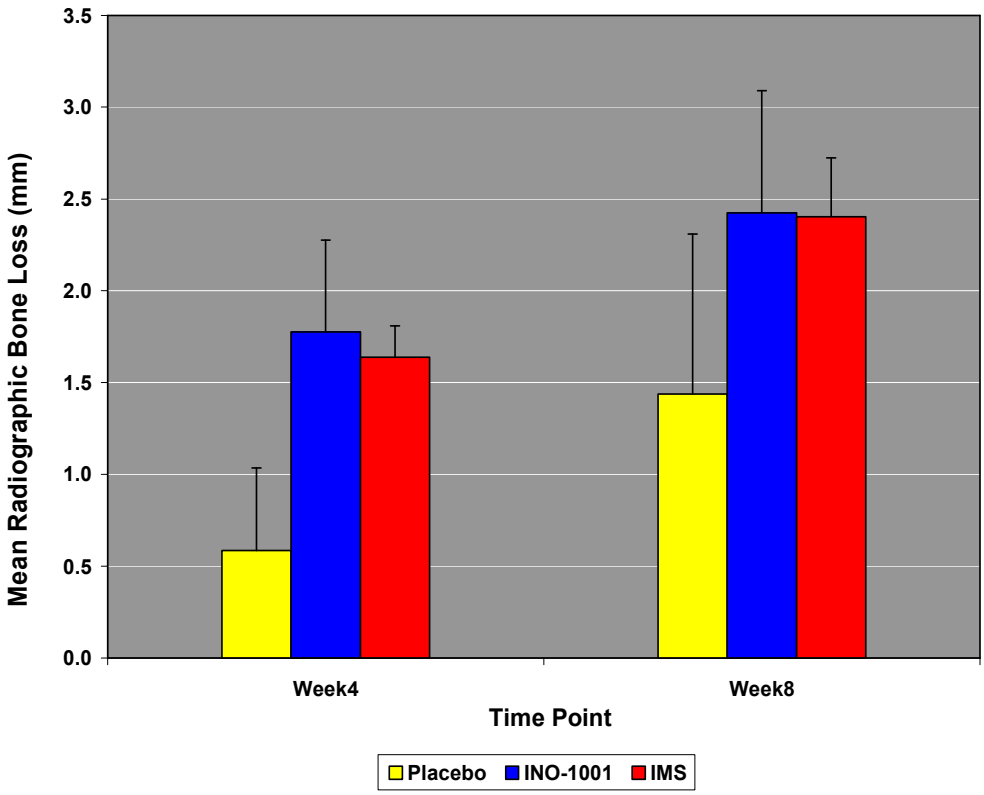


Figure 4

Phase II - Experimental Gingivitis Outcomes:
Mean Plaque Index (PI) Scores Stratified by Treatment Group and Time Point

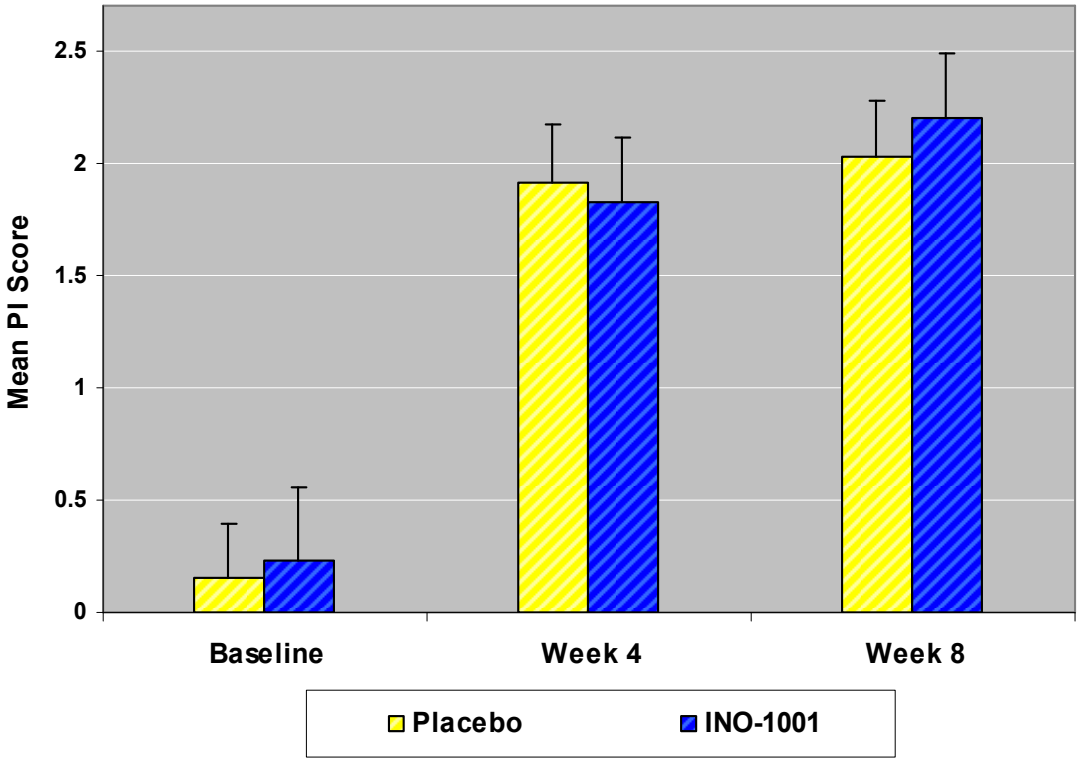
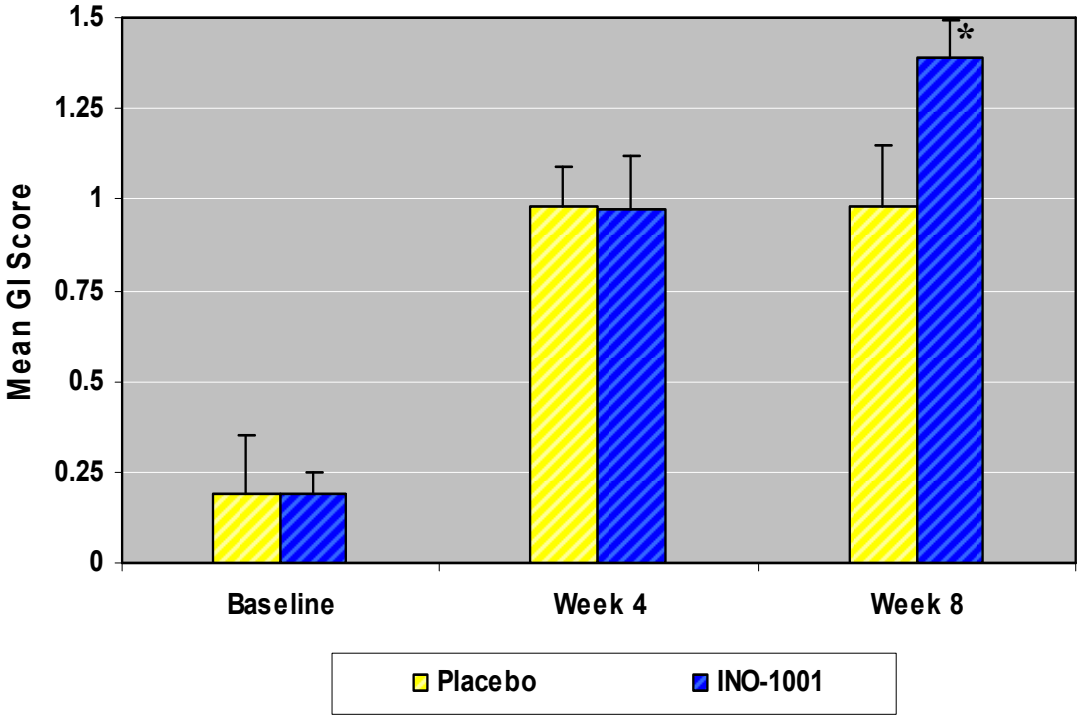


Figure 5

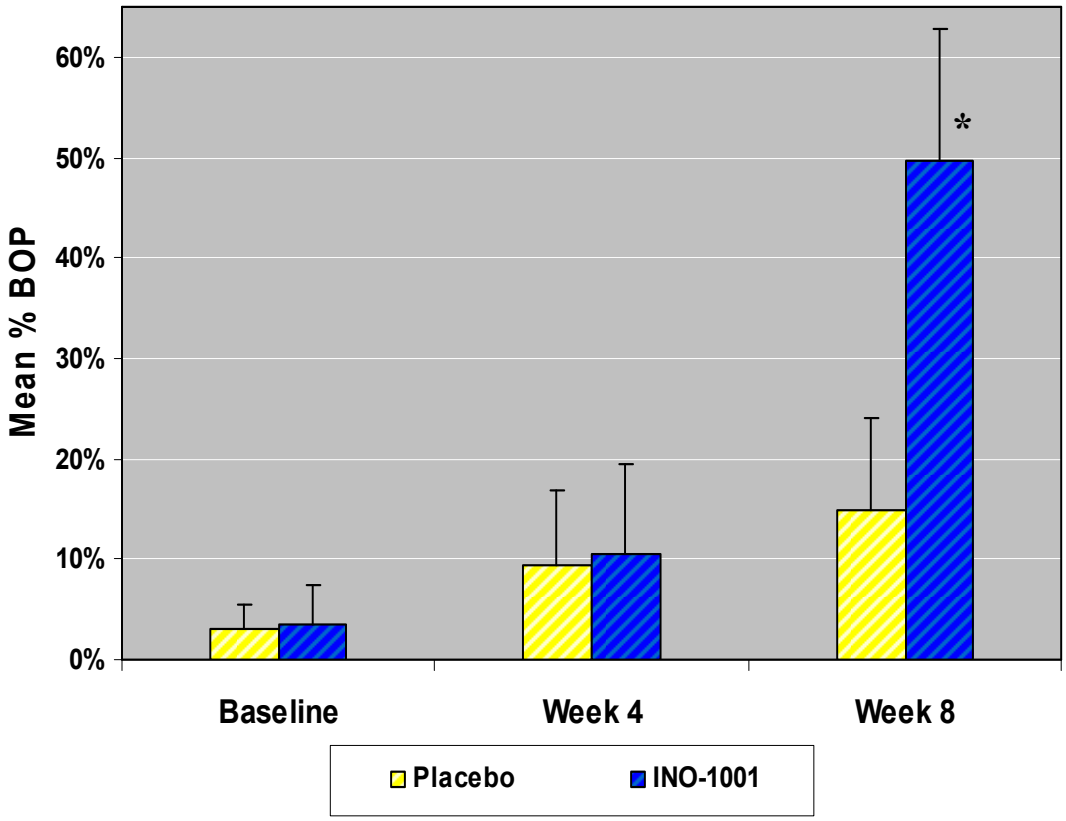
Phase II - Experimental Gingivitis Outcomes:
Mean Gingival Index (GI) Scores Stratified by Treatment Group and Time Point



* p< 0.0001 using General Linear Models (GLM)

Figure 6

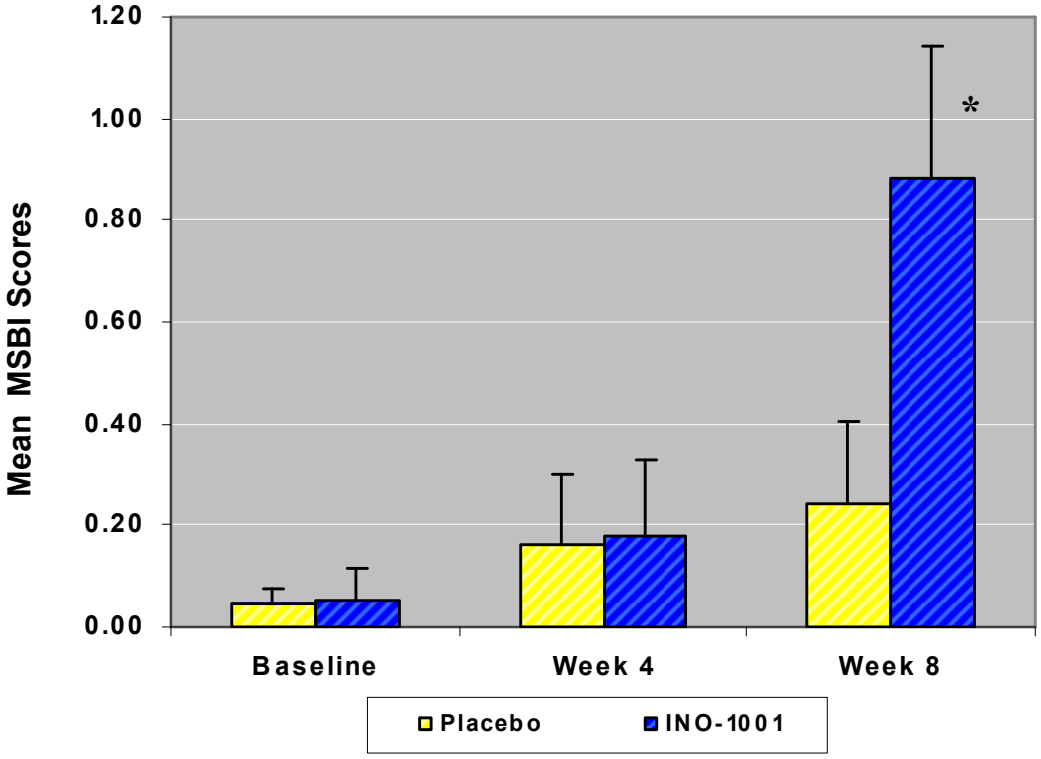
Phase II - Experimental Gingivitis Outcomes:
Mean Bleeding on Probing Scores Stratified by Treatment Group and Time Point



* p< 0.0001 using General Linear Models (GLM)

Figure 7

Phase II - Experimental Gingivitis Outcomes:
Mean Modified Sulcular Bleeding Scores Stratified
Group and Time Point



* p< 0.0001 using General Linear Models (GLM)

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